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(54) Title: COMPOSITIONS AND METHODS FOR CELL TRANSFORMATION

(57) Abstract

The present invention relates to methods and compositions for the transformation of cells. In particular, compositions and methods are disclosed which include combinations of the nucleic acid of interest and polyhydroxylated or polyglycosylated steroid molecules. Most preferably, exogenous or endogenous nucleic acid is contacted with the cell in the presence of a bile acid (e.g., cholic acid) derivatized with an amine-containing side chain.

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COMPOSITIONS AND METHODS FOR CELL TRANSFORMATION

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part (CIP) of co-pending U.S. Application Serial No. 08/336,675, filed November 7, 1994, which, in turn, is a CIP of co-pending U.S. Application Serial No. 08/264,488, filed June 23, 1994, which, in turn, is a CIP of co-pending U.S. Application Serial No. 08/230,685, filed April 20, 1994, which, in turn, is a CIP of co-pending U.S. Application Serial No. 07/989,667, filed December 14, 1992, which, in turn, is a CIP of U.S. Application Serial No. 07/806,985, filed December 13, 1991, now U. S. Patent No. 5,338,837, the disclosures of which are incorporated herein by reference.

1. FIELD OF THE INVENTION

The present invention relates to methods and compositions for the transformation of cells, which transformation involves the introduction of nucleic acid into eucaryotic and procaryotic cells. In particular, compositions and methods are disclosed which include combinations of the endogenous or exogenous nucleic acid of interest and polyhydroxylated or polyglycosylated steroid molecules, which include amine-containing groups that allow these molecules to be positively charged. The disclosed combinations include, preferably, at least one neutral lipid component, including, but not limited to, fatty acid esters, triglycerides, phospholipids, glycolipids, other steroid derivatives, such as cholesterol and cholesteryl esters, and lipoprotein complexes. The neutral lipid component is, preferably, a fusogenic lipid. As an example, the nucleic acid (e.g., exogenous nucleic acid) is contacted with the cell

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in the presence of a 1:1 mixture of dioleoyl phosphatidylethanolamine and a polyglycosylated cholic acid derivatized with a biogenic polyamine side chain.

2. BACKGROUND OF THE INVENTION

With the development of DNA recombinant methods and the advent of the biotechnology industry, methods for introducing nucleic acids into cells have been an ongoing focus of biologists and others in the field, partly because the efficiency of this process has remained quite low. For example, Friedmann, T. states in a review article, discussing progress toward human gene therapy and which appeared in Science (1989) 244:1275-1281, that efficiency of physical transfection methods *in vitro* can approach or exceed 1% in suitable recipient cells.

Research workers seeking to effect the transformation of cells, whether the cells are used in culture for the production of selected gene products or the cells form part of the organs or tissues of a living subject (i.e., in gene therapy), have resorted to a number of general strategies, including coprecipitation of the nucleic acid with inorganic salts, electroporation, direct injection of the oligonucleotide or use of cationic lipid/ nucleic acid mixtures.

Hence, Felgner, P.L., in Adv. Drug Deliv. Rev. (1990) 5:163-187, surveys the various methods for delivery of functional polynucleotides *in vitro* and *in vivo*, including the use of cationic polypeptides, diethylaminoethyldextran (DEAE dextran), calcium phosphate and other insoluble inorganic salts, liposomes, proteo-liposomes, and cationic lipids (e.g., N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride or DOTMA).

Liposomes have been a popular vehicle for introducing DNA into cells. For instance, Nicolau, C. et al., in Proc. Natl. Acad. Sci. USA (1983) 80:1068-1072,

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disclose the apparent *in vivo* expression of rat insulin in rat after i.v. administration of liposome-entrapped plasmid containing a sequence encoding rat preproinsulin I. Liposomes were made from a mixture of egg yolk phosphatidylcholine/ox brain phosphatidylserine/cholesterol, 8:2:10 (mol/mol/mol). These workers observed significant but transitory expression of insulin by liver and spleen cells.

Cationic liposome-mediated transfection (dubbed "lipofection") has been made popular by the work of Felgner and co-workers. The use of DOTMA in a DNA-transfection protocol has been described by Felgner, P.L. et al. Proc. Natl. Acad. Sci. USA (1987) 84:7417. The DOTMA is incorporated in dioleoyl phosphatidylethanolamine-based liposomes. DNA-total lipid complex is then prepared in HBS, which are then added to just-confluent cells. These authors claim to obtain 5- to >100-fold increased transfection over that shown for calcium phosphate on DEAE-dextran. In Focus (1989) 11(2):21-25, Felgner, P.L. and Holm, M. discuss the use of DOTMA, which is capable of forming liposomes and, importantly, capable of interacting spontaneously with DNA or RNA, to form a liposome/polynucleotide complex. A transfection reagent is described which can be used with a "wide variety of tissue culture cells and with different classes of polynucleotides including DNA, mRNA, dsRNA." Cells are incubated for 24-48 h at 37 °C. In Nature (1989) 337:387-388, Felgner, P.L. and Ringold, G.M. further discuss cationic liposome-mediated transfection using DOTMA. A schematic on p. 387 of this article shows the proposed structure of a liposome/nucleic acid complex. The authors note that one wants a net positive charge for the complex.

Mixtures of cationic lipid (DOTMA) and dioleoyl phosphatidy-lethanolamine (DOPE), commercially available as LIPOFECTIN™, and other cationic lipid-containing

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transfection-mediating compositions are described in articles by Brunette, E. et al. in Nucl. Acids Res. (1992) 20(5):1151 and Jarnagin, W.R. et al. in Ibid. (1992) 20(16):4205. Use of a mixture of lysinylphophatidylethanolamine (L-PE) and the cholesterol ester of beta-alanine (CEBA) is also disclosed in the latter article.

Malone, R.W. et al. in Proc. Natl. Acad. Sci. USA (1989) 86:6077-6081, describe the use of lipofection for cationic liposome-mediated RNA transfection. In this work, DOTMA was incorporated into liposomes with dioleoyl phosphatidylethanolamine (DOPE) in a 1:1 (mol/mol) ratio.

In an article appearing in Biotechniques (1988) 6(7): 682-670, Mannino, R.J. and Gould-Fogerite, S. discuss liposomes for use as "custom-designed cell-type specific gene transfer vehicles." These workers use large unilamellar vesicles (LUV) for high molecular weight molecules such as RNA and DNA (0.2-4.0 mm). The authors enumerate four characteristics that an efficient gene transfer vehicle should possess: (i) encapsulation of DNA; (ii) targeting and binding to target cells; (iii) fusion and delivery of liposome contents; (iv) nuclear targeting and expression. The liposomes were prepared from phosphatidylcholine and cholesterol.

Behr, J.P. et al., in Proc. Natl. Acad. Sci. USA (1989) 86:6982-6986, describe gene transfer experiments into mammalian primary endocrine cells using lipopolyamine-coated DNA. Certain lipocarboxyspermine derivatives are described which the authors contend mediate the successful transfection of a variety of eukaryotic cell cultures. The bacterial chloramphenicol acetyl-transferase (CAT) gene is used as the marker for gene transfer. The disclosure of this reference, and all others cited in the present application, is incorporated by reference herein.

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In addition, Leonetti, J.P. et al., in Proc. Natl. Acad. Sci. USA (1990) 87:2448-2451, discuss the use of antibody-targeted liposomes bearing oligonucleotides complementary to viral RNA. Encapsulated oligomers resist DNAse and are active in amounts 1-2 orders of magnitude lower than those reported for unencapsulated oligomer sequences. Liposomes are prepared from mixtures of dipalmitoyl phosphatidylcholine (65%), cholesterol (34% mol), and N-succinimidyl-3-(2-pyridyldithio)propionate-modified phosphatidylethanolamine (1% mol). The liposomes were conjugated to protein A and used in conjunction with protein A-binding monoclonal antibodies. Virus production was allegedly inhibited *in vitro*.

Juliano, R.L. and Akhtar, S., in Antisense Research and Development (1992) 2:165-176, focus on the use of liposomes as drug delivery systems for antisense oligonucleotides. The different types of liposomes of potential use are discussed, including small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), multilamellar vesicles (MLVs), and long circulation lifetime liposomes (LC-Lipos). So called fusogenic liposomes and antibody-conjugated liposomes are also described.

Complex mixtures involving DNA/protein and lipid/peptide complexes have very recently been utilized in DNA transfection protocols. In particular, Legendre, J.Y. and Szoka, Jr., F.C., in Proc. Natl. Acad. Sci. USA (1993) 90:893-897, discuss the use of "cyclic cationic amphipathic peptide gramicidin S and dioleoyl phosphatidylethanolamine." Certain combinations of DNA/peptide at 1:1 "charge ratio" and lipid/peptide at 5:1 "molar ratio" are disclosed. These workers boast transfection levels up to 20-fold higher than cationic liposomes in adherent mammalian cells and cite evidence that supports DNA entry into the cell via the plasma

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membrane. This work purports to address the need for improving transfection efficiency in non-viral systems. The article further suggests that the hydrophobic face of gramicidin S is important for transfection activity, diminishing, somewhat, the importance of charge neutralization. The authors allege that the phospholipids have two functions: to decrease the cytotoxicity of the gramicidin S; to enhance the transfection level.

Clark, P.O. and Leach F.R., in Molec. Gen. Genet. (1980) 178:21-25, have described the effect of millimolar concentrations of spermidine on the transformability of *Bacillus subtilis* cells. Maximum stimulation of the cells is observed when spermidine is added 30 minutes before DNA.

On the other hand, Gilbon, E. et al., in Biotechniques (1986) 4(6):504-511, discuss the utility of retroviral-mediated gene transfer for delivering a particular gene into a large fraction of a given cell population. Rosenfield, M.A. et al., in an article that appeared in Science (1991) 252:431-434, disclose the use of the Adenovirus genome, containing a cDNA expression cassette, for transfection of rat lung epithelium *in vivo*.

Lastly, Kaneda, Y. et al., in Science (1989) 243:375-378, observe increased expression of DNA cointroduced with nuclear proteins upon injection into the portal veins of adult rats. Expression was allegedly observed upon addition to cultural cells. The DNA and nuclear proteins were incorporated into Sendai virus-fused lipid vesicles.

In the general area of polyamine-steroid nucleus conjugates, a naturally-occurring spermidine-cholestanyl compound having antibiotic activity is described in U.S. Patent No. 5,192,756, granted to Zasloff et al. March 9, 1993. This compound was isolated from the stomach of the

common dogfish shark, *Squalus acanthias*. However, the specification contains no disclosure of any uses of this compound other than for its alleged antibiotic activity.

Bellini, A.M. and co-workers, in Arch. Pharm. (Weinheim) (1990) 323:201-205, have described the antimicrobial activity of basic cholane derivatives. These research workers found that hydrophobic amine derivatives possessed the highest antimicrobial activity. The mechanism of action was attributed to membrane crossing rather than receptor contact. This article states on page 205 that the *un*ionized species is the active species. Moreover, this article contains no disclosure, teaching or suggestion regarding nucleic acid transformation processes. Two earlier articles by Bellini and co-workers, in Eur. J. Med. Chem. (1983) 18(2):185-190 and Ibid. (1983) 18(2):191-195, described similar compounds and their antimicrobial activity. It is further noted that none of the derivatives disclosed included a polyamine chain that possessed at least one unsubstituted (i.e., $-NH_2$) amine group.

Burrows and co-workers have synthesized certain sterol dimers and trimers using amine spacers, as described in Abstract Nos. 193 and 304 from the 208th ACS National Meeting, Division of Organic Chemistry. These dimers and trimers are alleged to form cavities that bind to DNA.

Thus, there remains a need in the art for more effective ways of achieving transfection and, more generally, of introducing endogenous or exogenous nucleic acids into cells and, thus, affecting their genetic make-up.

3. SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method of introducing nucleic acid into cells by transformation. Broadly, the present

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invention comprises contacting the cells with the nucleic acid (exogenous or endogenous) in the presence of a compound, which comprises a bile acid-based molecule and at least one amine-containing moiety, preferably a polyamine. In one embodiment of the invention, a lipid, most preferably a neutral lipid, is also present in the contacting step. Optionally, the transfection medium also contains cationic lipids.

It has been discovered that the efficiency of transformation is increased in the presence of the compounds described herein compared with the transformation efficiency in the absence of same. Thus, the present invention can be used in a variety of applications, including, but not limited to, the facilitation of gene expression, protein engineering, protein production by a transformed host cell, cloning and subcloning procedures, antisense, gene therapies, and the like.

The present invention also seeks to provide the compounds which are of particular interest in achieving the above-noted objectives, as well as compositions comprising same. Thus, a further object of the present invention relates to the enhancement of the transformability of a host cell which includes contacting the host cell to be transformed with an effective amount of a compound of the invention or a composition containing same.

By using the compounds, compositions, and methods disclosed, it has been found that the introduction of nucleic acids into cells can be greatly facilitated. More importantly, the ability to deliver the nucleic acid can be extended potentially to those cells that comprise the tissues and organs of living organisms, with the consequent diagnostic, prophylactic, and therapeutic benefits associated therewith.

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Other objects of the present invention will become apparent to those of ordinary skill in the art upon further consideration of the detailed description of the preferred embodiments, presented below, which are meant to illustrate the basic concepts of the invention.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates the synthetic scheme for the preparation of 3α-p-methoxybenzoate-7α,12α-di(2,3,4,6-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic acid, methyl ester.

FIG. 2 illustrates the synthetic scheme for the preparation of 3β-amino-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic acid, methyl ester.

FIG. 3 shows additional examples of the types of aliphatic amine moieties suitable for conjugation to the bile acid compounds described herein.

FIG. 4 shows further examples of the types of aromatic amine moieties suitable for conjugation to the bile acid compounds described herein.

FIG. 5 illustrates the synthetic scheme for the preparation of 3α-hydroxy-7-deoxy-12α-(1'α-glucosyl)-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide (may also be referred to as the 12-(glycosylated)deoxycholic acid-spermine conjugate).

FIG. 6 illustrates the synthetic scheme for the preparation of 3α-hydroxy-12-deoxy-7α-(1'α-glucosyl)-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide (may also be referred to as the 7-(glycosylated)chenodeoxycholic acid-spermine conjugate).

FIG. 7 illustrates the synthetic scheme for the preparation of 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide (may also be referred to as the cholic acid-spermine conjugate, Compound F).

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FIG. 8 illustrates the synthetic scheme for the preparation of 3α,12α-dihydroxy-7α-deoxy-5β-cholan-24-oic acid, N-(12-aminododecyl)amide (may also be referred to as the deoxycholic acid-1,12-diaminododecane conjugate, Compound G).

FIG. 9 illustrates the synthetic scheme for the preparation of 3α-hydroxy-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide (may also be referred to as the bis(glycosylated)cholic acid-spermine conjugate, Compound E).

FIG. 10 illustrates additional deoxycholic acid- and chenodeoxycholic acid-poly(aminoalkylene) conjugates of the present invention.

FIG. 11 illustrates the synthetic scheme for the preparation of 3β-amino-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide trihydrochloride.

FIG. 12 lists some of the compounds that were used for the transformation of COS-7 cells.

FIG. 13 illustrates the synthetic scheme for selected compounds 2 and 5 of FIG. 12.

FIG. 14 illustrates the synthetic scheme for the synthesis of compounds 6 and 7 of FIG. 12.

FIG. 15 is a schematic representation of the pSVβ used in the transformation experiments.

FIG. 16 is a table listing some of the results of the transformation experiments.

FIG. 17 is a graphical representation of the transformation efficacy relative to compound:DNA charge ratio.

FIG. 18 is a table comparing results for the ONPG and X-GAL assays for various compound M/DOPE complex to DNA charge ratios.

FIG. 19 illustrates the transfection efficiencies of Lipofectin, compound E, and compound M for the

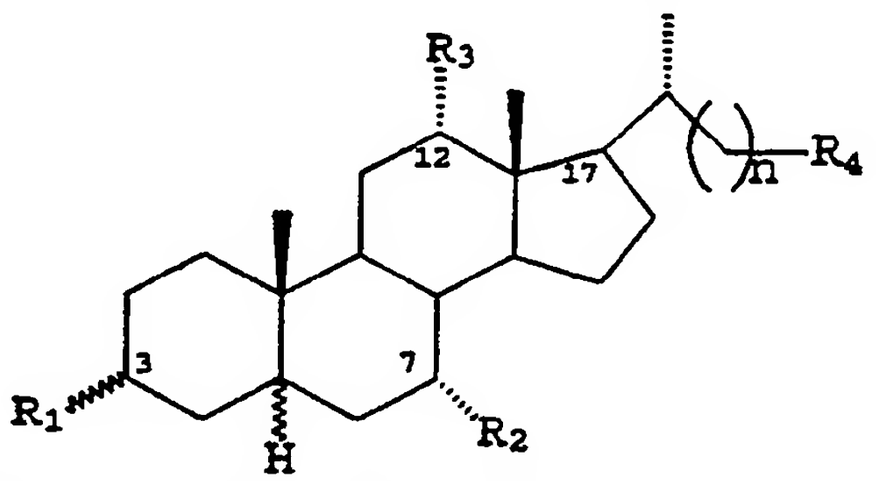
transfection of COS-7 cells with pSVβ plasmid under phase contrast and bright field conditions.

FIG. 20 illustrates the effect of the addition of 1% serum on the transfection of COS-7 cells with pSVβ plasmid in the presence of compound M/DOPE complex at various compound M to DNA charge ratios.

FIG. 21 presents the results of gel shift experiments using DNA alone (control), compound 1, spermine alone, and compound E, which is the spermine derivative of compound 1.

5. DETAILED DESCRIPTION OF THE INVENTION

The compounds, compositions, and methods of the present invention may be applied for the advantageous introduction of nucleic acid, particularly exogenous nucleic acid, to a cell. In particular, the present method for the introduction of exogenous nucleic acid to a cell comprises: (a) contacting a cell with nucleic acid in the presence of a compound of the formula (I):



(I)

in which R_1 can be an H, OH, OR_5, NH_2, NHR_6 or NR_6R_7; R_2 and R_3 may be the same or different and can be an H, OH or OR_5; R_4 can be $CONH_2$, $CONHR_6$, $CONR_6R_7$, CH_2NH_2, CH_2NHR_6, $CH_2NR_6R_7$, CO_2-Y-NH_2, CO_2-Y-NHR_6, or CO_2-Y-NR_6R_7; R_5 is a protected or unprotected glycosyl moiety comprising 1-10 monosaccharide units in which the glycosidic linkage at the anomeric carbon atom of each monosaccharide unit is independently alpha or beta; NH_2, NHR_6, and NR_6R_7 represent an unsubstituted amino group, monosubstituted amino groups, and a disubstituted amino group,

respectively, in which R_6 and R_7 may be the same or different and represent a linear, branched or cyclic hydrocarbon group (e.g., an aliphatic group, a cyclic aliphatic group, an aromatic group or combinations of same) comprising 1-15 carbon atoms optionally substituted with one or more unsubstituted, monosubstituted or disubstituted amino groups; Y represents a linear or branched alkylene group comprising 1-10 carbon atoms; n is an integer from 0-10, preferably 0-3; or its acid addition or quaternary ammonium salt; and (b) allowing the nucleic acid to remain in contact with the cell in the presence of the compound for a period of time sufficient to effect the introduction of the nucleic acid to the cell.

The degree of substitution of the amino group is determined by the number of bonds to hydrogen emanating from the amino group. Thus, an unsubstituted amino group has two N-H bonds (e.g., $-CH_2-CH_2-NH_2$). A monosubstituted amino group has one N-H bond (e.g., $-CH_2-NH-CH_2-$ or $-CH=NH$). A disubstituted amino group has none (e.g., $=CH-NR-CH_2-$ or $-CH=N-CH=$). By "substituted with one or more unsubstituted, monosubstituted or disubstituted amino groups" is meant that the hydrocarbon group comprising 1-15 carbon atoms contains at least one amino group either within the hydrocarbon backbone (e.g., $-CH_2-NH-CH_2-$, $-CH_2-NR-CH_2-$, $-CH=N-CH_2$, $-CH=N-CH=$, and the like) or coming off the backbone (e.g., a primary amine, a secondary amine, a tertiary amine, an imine or the like, such as $-CH_2-CH_2-NH_2$, $-CH_2-CH(-NH_2)-CH_2-$, $-CH_2-CR(NH_2)-CH_2-$, $-CH=NH$ or $-CR=NH$).

Accordingly, such amino groups are capable of accommodating a charge, for example, in protic media (e.g., $-CH_2-NH_2^{\oplus}-CH_2-$ or $-CH_2-CH_2-NH_3^{\oplus}$) or on formation of a quaternary ammonium salt (e.g., $-CH_2-CH_2-NMe_3^{\oplus}$, wherein Me stands for methyl). The preferred compounds of the present invention include those that are able to

accommodate two or more positive charges. Yet others can accommodate three, four or even more positive charges.

Additional examples of selected amino group-containing moieties, that may be used as R6 and/or R7, can be found in Figures 3 and 4.

As stated above, the group R_5 can be a protected or unprotected glycosyl moiety, which, in turn, may comprise 1-10 monosaccharide units (e.g., a monosaccharide, a disaccharide, a trisaccharide, etc.). In the present case, the term "monosaccharide" is any sugar residue or derivative thereof. The monosaccharide may, for example, be a hexose (e.g., D-allose, L-allose, D-altrose, L-altrose, D-fucose, L-fucose, D-glucose, L-glucose, D-mannose, L-mannose, D-gulose, L-gulose, D-idose, L-idose, D-galactose, L-galactose, D-rhamnose, L-rhamnose, D-talose, L-talose, and the like, or any deoxy form thereof, e.g., a 2-deoxyhexose, or any amino-substituted derivative thereof, e.g., an aminosugar, such as D-glucosamine, L-glucosamine, D-galactosamine, L-galactosamine, etc.). Furanoses, deoxyfuranoses, amino-substituted furanoses, and the like are also suitable, such as D-ribose, L-ribose, D-arabinose, L-arabinose, D-xylose, L-xylose, D-lyxose, L-lyxose, etc.

Furthermore, the protecting groups for the hydroxyl groups (or amino groups, as the case may be) can be chosen from a wide variety of protecting groups appropriate for a given set of conditions. These protecting groups, the choice of which will be apparent to one skilled in the art, may include, but are not limited to, benzyl, pentenyl, pivaloyl, trimethylsilyl, tert-butyldimethylsilyl, tert-butyldiphenylsilyl, triisopropylsilyl, acetyl, tetrahydropyranyl, benzoyl, C_1-C_3 alkyl, isopropylidene, benzylidene, trifluoroacetyl, (2-methoxyethoxy)methyl, succinyl, orthoester, paramethoxybenzyl, allyl, and the like.

The acid addition or quaternary ammonium salt of the conjugates of interest are preferably pharmaceutically acceptable. Examples of such pharmaceutically acceptable salts include, but are not limited to, inorganic and organic addition salts, such as hydrochloride, sulphates, nitrates or phosphates and acetates, trifluoroacetates, propionates, succinates, benzoates, citrates, tartrates, fumarates, maleates, methane-sulfonates, isothionates, theophylline acetates, salicylates, respectively, or the like. Lower alkyl quaternary ammonium salts and the like are suitable, as well.

In the present method it is contemplated that the nucleic acid introduced, or at least a portion thereof, leads to its incorporation within the genetic make-up of the cell. Such incorporation may, for example, result in the integration of the nucleic acid, or at least a portion thereof, within a chromosome of the cell. In particular, the segment of nucleic acid may become inserted into a region of a chromosome or may even displace selected endogenous sections thereof. Also, the added nucleic acid, or at least a portion thereof, may be retained by the cell as extrachromosomal material.

In a specific embodiment of the present invention, the method includes contacting the cell with the exogenous or endogenous nucleic acid in the presence of a compound of the formula (I) and, further, in the presence of a lipid or lipids. Preferably, the lipid is polar and, most preferably, is a fusogenic lipid. In the present invention, the term "fusogenic" refers to a property or characteristic that allows the fusogenic material, e.g., a lipid, to promote the fusion of the nucleic acid/bile acid-poly(aminoalkylene)/lipid complex with the cell membrane, such that substances initially located in the exterior of the membrane may eventually penetrate and proceed through to the interior of the membrane. Examples of fusogenic lipids include certain

phosphatidylethanolamine head group-containing phospholipid, e.g., DOPE, DMPE. In particular phospholipid compounds, their fusogenic behavior has been correlated with their ability to undergo a bilayer-to-hexagonal phase transition.

The present invention is not limited to the use of such compounds, however, and any lipid that displays fusogenic behavior may be used herein. For example, lysophospholipids, such as lysinyl phosphatidylethanolamine may also promote the fusion of DNA-bile acid amine-lipid complexes. Other general classes of lipids, in addition to phospholipids, lysophospholipids, and fatty acid esters or ethers of sugars, include glycosyl diacylglycerols (e.g., monogalactosyl-diglyceride), plasmalogens (e.g., ethanolamine plasmalogen), glycosphingolipids, including cerebrosides, gangliosides, sterols, and diphosphatidylglycerols (e.g., cardiolipin-Ca^{2+}). Several of these classes of lipids contain members known to form either hexagonal phases or micellar structures.

In still other embodiments, a cationic lipid may also be present in the composition. Hence, lipids bearing quaternary ammonium groups are also contemplated for use in the present invention. Accordingly, examples of cationic lipids include, but are not limited to, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride (DOTMA), 1,2-dimyristoyl-3-trimethylammonium propane (DOTAP), and 1,2-dimyristoyl-3-dimethylammonium propane (DODAP).

General groups of neutral lipids suitable for use in the present invention include, but are not limited to, phospholipids such as DOPE, lysophospholipids such as L-PE, and fatty acid esters such as sucrose monooleate and sucrose monolaurate. Other general groups of potential use in the present invention are glycosyl

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diacylglycerols, plasmalogens, aphingomyelins, gangliosides, glycerolipids, sphingolipids or cardiolipins.

In a particular embodiment, a fusogenic lipid with a net neutral charge (e.g., DOPE) is optionally combined with a net positive charge (e.g., DOTMA) and subsequently added to a mixture of a compound of the present invention and the nucleic acid or acids of interest. In specific embodiments of the invention, the contacting step is carried out further in the presence of diethylaminoethyldextran (DEAE) or the like.

Further, it may be desirable in some instances to pre-mix the neutral lipid and the compound of the formula (I) with the nucleic acid to allow for the formation of a complex between the nucleic acid and the compound. The resulting mixture is then allowed to contact the cell into which the nucleic acid is to be transformed or introduced.

Thus, the present invention is also directed to a particular group of compounds. Indeed compounds of the formula (I) are disclosed in which R_1 can be an H, OH, OR_5, NH_2, NHR_6 or NR_6R_7; R_2 and R_3 may be the same or different and can be an H, OH or OR_5; R_4 can be $CONHR_6$, $CONR_6R_7$, CH_2NHR_6, $CH_2NR_6R_7$, CO_2-Y-NH_2, CO_2-Y-NHR_6, or CO_2-Y-NR_6R_7; R_5 is a protected or unprotected glycosyl moiety comprising 1-10 monosaccharide units in which the glycosidic linkage at the anomeric carbon atom of each monosaccharide unit is independently alpha or beta; NH_2, NHR_6, and NR_6R_7 represent an unsubstituted amino group, a monosubstituted amino group, and a disubstituted amino group, respectively, in which R_6 and R_7 may be the same or different and represent a linear, branched or cyclic hydrocarbon group comprising 1-15 carbon atoms substituted with one or more unsubstituted, monosubstituted or disubstituted amino groups, provided that one of which R_6 or R_7 must include at least one unsubstituted (preferably, primary) amino group; Y

represents a linear or branched alkylene group comprising 1-10 carbon atoms; n is an integer from 0-10, preferably 0-3; or its acid addition or quaternary ammonium salt.

In a specific embodiment, the group R_1 has the configuration beta. In another, the group R_1 has the configuration alpha. In a particular embodiment, at least one of R_1, R_2, and R_3 represents OH. In another embodiment, at least two of R_1, R_2, and R_3 represent OH, and in still another embodiment, all three of R_1, R_2, and R_3 represent OH.

The present invention contemplates all other combinations of the various groups, including, but not limited to, embodiments in which R_1 and R_2 represent OR_5, and R_3 represents OH; R_1 and R_3 represent OR_5, and R_2 represents OH; or R_2 and R_3 represent OR_5, and R_1 represents OH.

Furthermore, a compound is disclosed in which the group R_6 together with the nitrogen atom to which it is attached derives from a polyamine. Suitable polyamines include, but are not limited to, alkylene diamines, such as 1,3-diaminopropane, and biogenic polyamines, such as 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), N-(4-aminobutyl)-1,3-diaminopropane (spermidine, an alkylene triamine), and N-[N-(3-aminopropyl)-4-aminobutyl]-1,3-diaminopropane(spermine, an alkylene tetra-amine), and the like, including branched aliphatic polyamines. With unsymmetrical polyamines, the present invention contemplates all other possible points of attachment of the polyamine to the steroid nucleus. For example, in spermidine, any of the three amino groups may be attached to the side chain or at the C-3 position of the steroid nucleus.

In selected embodiments of the present invention, the group R_1 or R_4 is neither an amino acid nor a peptide.

The nucleic acid to be introduced to the cell can comprise DNA or RNA and can take many forms. For example, the nucleic acid may be single-stranded, double stranded (or contain both single-stranded and double-stranded regions, as in a ribozyme), may comprise chromosomes, fragments thereof, plasmids, phage-derived or may be contained in vectors, such as cloning vectors, expression vectors, and yeast artificial chromosomes. Preferably, the nucleic acid encodes a gene and, most preferably, a gene of mammalian or plant origin. In a specific embodiment, the nucleic acid is an oligo- or polynucleotide, preferably an antisense sequence. For example, the antisense sequence may correspond to a splice acceptor site or its complement, e.g., the sequence 5' ACA CCC AAT TCT GAA AAT GG 3' or its complement. Alternatively, the oligonucleotide has a sequence corresponding to a primer binding site or its complement. Such a sequence may include, for example, the sequence 5' AAG TCC CTG TTC GGG CGC CA 3' or its complement.

The preferred steroidal nucleus includes, but is not limited to, bile acids, cholic acid, allocholic acid, 3β- and 3α-amino-5β-cholic acid, lithocholic acid, deoxycholic acid, chenodeoxy cholic acid or 3-deoxycholic acid. Cholestanyl derivatives may also be used but are less desirable, particularly those that may contain negatively charged groups.

Accordingly, the present invention has tremendous promise not only in the areas of gene expression, protein manufacturing, and the like, but also in the diagnostic, prophylactic, and therapeutic areas, particularly in antisense and gene therapies. By enhancing the uptake of transforming nucleic acid, the efficiency of transformation is increased. Consequently, the dosing levels may also benefit from the instant invention.

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5.1 Description of Typical Results

Various assays have been performed which confirm the formation of a tight complex between DNA and the compounds of the present invention, the successful transfection of cells, and the expression of the DNA-encoded protein in the transfected cells. Among these assays, the staining assay and the onpg assay both rely on the reporter gene β-galactosidase (β-gal), which is present in the plasmid DNA used for the transformation experiments. The agarose gel assay provides an indication of the efficiency of complex formation.

5.2. Agarose Gel Assay to Evaluate Complex Formation

Typically, it has been found that preferred compounds or mixtures for facilitating uptake of DNA into cells form complexes with DNA. To evaluate the ability of various compounds to complex DNA, an agarose gel assay is used. Compounds that form tight complexes with DNA cause the DNA to be retained in or near the well at relatively low (i.e., less than 10) charge ratios (the charge ratio is the ratio of positive charges in the bile acid conjugates to negative charges in the DNA, assuming complete ionization of all the amine groups of the bile acid conjugates).

In a typical assay, 250-500 ng of pBR322 plasmid DNA are mixed with varying amounts of the bile acid conjugates or mixtures of the bile acid conjugates with other lipids in a total volume of 10 μL and incubated for 30 minutes. To each sample is then added 2 μL of 6X ficoll loading buffer and the samples are loaded on a 1% horizontal agarose gel prepared with 0.5X TBE. Horizontal gel electrophoresis in 0.5X TBE is then carried out for 2-3 hours at 100-125 volts.

The gel is then removed from the electrophoresis chamber and stained with ethidium bromide (5 μg/mL). The gel is placed on a transilluminator and photographed.

The composition of the 6X ficoll loading buffer and the TBE running buffer can be found in Sambrook, J.: Fritsch, E. F.; and Maniatis, T., *Molecular Cloning*, Cold Spring Harbor University Press: Cold Spring Harbor, 1989.

A number of compounds and compositions can be tested in this manner, including those listed in Table 1, below.

Table 1: Gel Retardation Assay Results

		Charge ratio DNA:compound	
Compound	1:1	1:2.5	1:5
A:B 1:1	partially retarded	partially retarded	fully retarded
B alone	partially retarded	partially retarded	fully retarded
A:B 1:3	partially retarded	partially retarded	fully retarded
A:C 1:1	no retardation	partially retarded	partially retarded
C alone	no retardation	no retardation	no retardation
A:D 1:1	no retardation	no retardation	no retardation
D alone	no retardation	no retardation	no retardation
A:E 1.25:1	no retardation	partially retarded	fully retarded

Key to abbreviations:
Compound A = DOPE (dileoyl phosphtidylethanolamine)
Compound B = Deoxycholic acid-spermine conjugate
Compound C = Deoxycholic acid conjugated, via amide side chain, to: $-NHCH_2CH_2N(CH_3)_3$
Compound D = 3β-amino-7α,12α-di(1'α-glucosyl)-5β-cholic acid, methyl ester
Compound E = Bis(glycosylated)cholic acid-spermine conjugate, via amide side chain

5.3. Materials

Cos-7 and (10)3 cells are cultured in D-MEM with 10% Fetal Bovine Serum (FBS) containing 100 units/mL penicillin G sodium and 100 μg/mL streptomycin sulfate. All cell culture media and components can be purchased from Gibco-BRL.

Plasmid pSV40-βGal, obtained from Promega, is amplified in NovaBlue cells (Novagen) and isolated and purified by the alkaline lysis method followed by acidified phenol extraction. Aubin, R.; Weinfeld, M.; Paterson, M. C., Chapter 1 in *Methods in Molecular Biology, Volume 7: Gene Transfer and Expression Protocols*, Murray, E.J., ed., Humana Press: Clifton, NJ 1991.

5.4. Transient Transfection of Cells

Cells are plated the day before transfection in 35 mm wells. The number of cells plated/well is approximately 3 x 10^5. Prior to transfection, the cells are washed three times with phosphate-buffered saline, pH 7.2 (Gibco-BRL). The cells are then overlaid with 1 mL of an Opti-MEM solution (Gibco-BRL) containing 2 μg of pSV40-β-Gal plasmid and varying amounts of the test compounds or compositions (e.g., bile acid-conjugates or a mixture of the bile acid-conjugates and lipid, DOPE). The cells are incubated for 3 hours at 37 °C in a humidified atmosphere containing 5% CO_2 and then 1 mL of 20% FBS/D-MEM *without* added antibiotics is added to each well. The cells are incubated for another 25 hours and then assayed for expression of β-galactosidase.

In experiments summarized in Tables 3 and 4, transfection mixtures contain 1 μg/mL of DNA in a volume of 204 μL. DNA:enhancer mixtures are initially formulated as 5-fold concentrates, incubated for 15 minutes, diluted to their final concentration in Opti-MEM without serum, and then applied to cells. Assays are performed in 11.3 mm wells of a 24-well plate containing 2 x 10^4 cells/well. Cells are exposed to transfection mixtures for 6 hours, after which the transfection mixture is removed. The plates are re-fed with D-MEM containing 10% fetal bovine serum. β-galactosidase expression is examined after 48 hours incubation.

5.5. In Situ Staining of Transfected Cells

Cells are rinsed with PBS and fixed for 10 minutes in 0.5% glutaraldehyde. They are then rinsed twice with PBS and stained overnight in 1 mL of a solution containing: 5 mM K^+ ferricyanide (Sigma); 5 mM K^+ ferrocyanide (Sigma); 1 mM $MgCl_2$, 1 mg/mL X-gal (Boehringer-Mannheim).

Cos-7 cells are treated as described with a 2 μg of pSV40-β-gal DNA in the presence of 6 μL of a 5 mg/mL solution of a 1:1 (w:w) deoxycholate-spermine conjugate:DOPE. The results of the staining assay are summarized in Table 2, below.

Table 2: Summary of Qualitative Transfection Efficiency

COMPOUND	ORIGINAL CONCENTRATION	VOLUME[a]	TRANSFECTION EFFICIENCY
LIPOFECTIN	1 mg/mL	4-8 μL	+++++++
A:B 1:1	5 mg/mL	4-8 μL	+++++++
B alone	2.5 mg/mL	8 μL	+
A alone	2.5 mg/mL	[b]	-
A:E 1.25:1	5 mg/mL	16 μL	+++
A:C 1:1	5 mg/mL	[b]	+
A:D 1:1	5 mg/mL	[b]	-
A:B 1:3	5 mg/mL	4-8 μL	++
A:B 3:1	5 mg/mL	4-8 μL	++
A:spermine	5 mg/mL	12 μL	++

[a]Range in the volume of the solution containing the original concentration found to provide optimal transfection efficiency under the conditions reported above.

[b]The results indicate no obvious difference at all volumes tested.

A separate set of experiments using slightly different conditions is further carried out. The data are presented in Table 3. The data are calculated based on the counted number of transfected cells within either the total complete well, or the average of five 100X fields across the well. Transfections are performed with 1 μg/mL of DNA, and the cells are stained 48 hours following the incubation with DNA. In all cases, the ratio of enhancer to lipid is held at 1:1 (w:w).

Table 3: Cell Staining by X-GAL

Compound	Concentration[a]	% Transfection[b]
LIPOFECTIN	8 μg/mL	100
A	8 μg/mL	0
DISTAP:A (1:1)	8 μg/mL	32.4 (10.5 - 59.2)
F alone	16.5 μg/mL	0.7 (0.09 - 1.3)
F:A (1:1)	16.5 - 33 μg/mL	36.8 (15.0 - 59.1)
G alone	12.5 μg/mL	1.0 (0.5 - 1.4)
G:A (1:1)	12.5 μg/mL	10.7 (4.8 - 16.6)
E	36 μg/mL	0.6 (0.3 - 0.9)
E:A (1:1)	36 μg/mL	216 (93 - 339)
B alone	16.5 μg/mL	0
B:A (1:1)	16.5 - 33 μg/mL	393.5 (188 - 599)

[a]Concentration of each component in transfection mixture to produce highest observed transfection efficiency.
[b]Average of experiments (range) as a percentage of LIPOFECTIN control.

Key to Abbreviations (See, Table 1 for others):
DISTAP = 1,2-Distearoyl-3-trimethylammoniumpropane

E = 3α-Hydroxy-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic acid, N'-(4,9-diaza-12 aminodecane)amide (same as in Table 1)

F = Cholic acid-spermine conjugate

G = 3α,12α-Dihydroxy-7-deoxy-5β-cholan-24-oic acid, N'-(12-amino-n-dodecyl)amide

The data in Tables 2 and 3 demonstrate that the compounds with the spermine side chain (e.g., B, E, and F), when mixed with DOPE and DNA, transfect cells at frequencies that approach, and in some cases, exceed that observed for LIPOFECTIN and cationic:neutral (DISTAP:DOPE) lipid mixtures.

5.6. **Assay For Expression Levels, ONPG Assay**

Table 4, below, provides the results of this quantitative measure of transfection efficiencies. In this assay, the cells are grown as described for the staining assay (see, Sections 5.3 and 5.4, above) and then treated as follows:

Preparation of cell lysate: The growth medium is removed from the cells to be assayed. The cells are washed twice with PBS buffer. The PBS buffer is removed. To the cells is added enough 1X Reporter Lysis Buffer (Promega) to just cover the cells (~250 μL for a 35 mM culture well). The dish is then incubated at room temperature for 15 minutes. The cell lysate is then transferred to a microfuge tube (a cell scraper is required to loosen all cell debris) with a pipet, and the tube is placed on ice. The tubes from the various wells are then vortexed for 15 seconds and centrifuged at top speed in a microcentrifuge (~14K rpm) for 2 minutes at 4 °C. The supernatant is transferred to a fresh tube and either frozen at -70 °C or used immediately.

Assay of β-gal activity: 150 μL of cell extract is placed in a microfuge tube (as a control, the same amount

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of cell extract from cells that have not been transfected with the β-gal gene is placed in another tube). 150 μL of Assay 2X Buffer (Promega) is added to the tube. The Assay 2X Buffer contains ONPG, which is a substrate for β-gal. The sample is vortexed and incubated at 37 °C for 30 minutes. The reaction is stopped by adding 500 μL of 1M sodium carbonate and vortexing. The absorbance is then read at 420 nm against the cell extract. In order to determine the β-gal activity, the absorbance is multiplied by 380 and divided by the incubation time (in minutes). 1 U = 1 nmol of ONPG hydrolyzed/min at 37 °C. (One can also make a calibration curve using a pure preparation of β-galactosidase.)

Table 4: Units of β-Galactosidase

Composition (μL)*	Exp. 1	Exp. 2	Exp. 3
LIPOFECTIN (4)	7.06	3.04	3.08
LIPOFECTIN (7)	6.25	7.8	5.39
A:B 1:1 (4)	27.217	9.67	-
A:B 1:1 (8)	4.638	4.2	7.47
A:B 1:3 (2)	1.71		
A:B 1:3 (4)	0.953		
A:B 3:1 (8)	0.684		
A:B 3:1 (16)	0.98		

* The original concentrations used are the same as those shown in Table 2, above

Table 4 shows that A:B 1:1 is better than LIPOFECTIN (which is a 1:1 mixture of DOTMA and DOPE) in effecting transformation of cells. There is variability between assays but the general trends hold up and are consistent with the staining results.

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Under slightly different conditions, the ONPG assay is again used to determine relative β-galactosidase activity as a measure of transfection efficiency. The data presented in Table 5 are determined by *in situ* analysis of the wells of the 24-well transfection plates. Cells are rinsed three times with PBS and freeze-thawed three times in distilled water. The β-galactosidase activity of the lysates is determined by the addition of an equal volume of substrate mixture that gives a final concentration of 0.88 mg/mL ONPG, 1 mM $MgCl_2$, 45 μM β-mercaptoethanol, in 100 mM sodium phosphate buffer pH 7.5. The assay plate is incubated at 37 °C for 30-60 minutes. A 100 μL aliquot of the reaction mixture is transferred to a 96-well plate. The 96-well plate is read in a microplate reader at 420 nm. A calibration curve of β-galactosidase, incubated with the 24-well plates, is included on the 96-well plate. The β-galactosidase activity is determined from the equation derived by linear regression of the calibration curve.

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Table 5: Relative Activity of β-galactosidase

Compound	Concentration[a]	Rel. Enzyme Activity (%) [b]
LIPOFECTIN	8 μg/mL	100
DISTAP:A(1:1)	8 μg/mL	37.5 (16.4 - 84.2)
F alone	2.2 μg/mL	12.9 (10.4-15.4)
F:A (1:1)	11 - 22 μg/mL	36.4 (26.5 - 46.3)
G alone	16.7 μg/mL	3.1 (2.9 -3.4)
G:A (1:1)	8.3 - 16.7 μg/mL	12.5 (11.4 -13.6)
E	6 - 12 μg/mL	6.9 (3.3 - 10.6)
E:A (1:1)	24 μg/mL	126.6 (77.9 - 175.4)
B alone	11 μg/mL	17.9
A:B (1:1)	11 - 22 μg/mL	430.4

[a]Concentration of each component in transfection mixture to produce highest observed transfection efficiency.

[b]Average of experiments (range) as a percentage of lipofectin control.

The compound abbreviations are identical to those used above.

The data in Tables 4 and 5 demonstrate that the compounds with the spermine side chain (e.g., A, E, and F), when mixed with DOPE and DNA, promote transfection at frequencies that approach, and in some cases, exceed that observed for lipofectin and cationic/neutral (DISTAP:DOPE) lipid mixtures. The relative efficacy found for the various compounds based on the enzyme assay closely parallels those found using the X-gal staining assay. These results suggest that the compounds of the

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invention primarily increase the number of cells that become transfected. It should be emphasized, however, that the data presented in Tables 4 and 5 have not been optimized and results approaching those observed for the A:B (1:1) experiments are anticipated for the closely related analogs of B, such as E or F, for example.

Thus, the compounds and compositions described herein provide expression levels (and, hence, transfection levels) that meet or exceed those observed for currently available commercial transfection agents. Moreover, it should be pointed out that the compounds and compositions disclosed herein may prove less toxic to the host cells (or to individuals, organs, tissues, etc.) relative to currently available agents.

As a further illustration of the present invention, examples of preferred embodiments are presented below.

6. EXAMPLES

6.1. 2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranose (2)

Methyl-α-D-glucopyranose (100 g, 0.516 mol) is suspended in benzyl chloride (400 mL, 3.5 mol) with KOH pellets (336 g, 6 mol), and the mixture is stirred using a mechanical stirrer at 120-130 °C for 3 h, as shown in Fig. 1. The reaction mixture is cooled and water (800 mL) is added to dissolve the crystalline mass, which is extracted with ether (2 x 200 mL). The combined organic layer is washed with water (2 x 500 mL) and dried (Na_2SO_4). The solvents are removed by vacuum distillation to give the crude methyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyrano-side for the next reaction.

To a stirred solution of above crude compound in glacial acetic acid (700 mL) at 110 °C is added 3N sulfuric acid (120 mL) dropwise during 15 min. After 3 h the reaction mixture is cooled to room temperature and left over night for crystallization of product. The crystals are filtered, washed consecutively with water (4

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x 500 mL) and methanol (2 x 250 mL), and air dried to afford **2** (115 g, 41% overall two steps) as a white powder (mp 150-51 °C, Lit. 151-152 °C; See, Perrine, T. D. et al. *J. Org. Chem.* (1967) 32:664). TLC (EtOAC:Hexane 3:7) Rf 0.2. IR (KBr): 3362, 3030, 2911, 2863, 1454, 1357, 1146, 1088 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.38-7.10 (m, 20H), 5.21 (d, J = 3.3Hz, 1H), 4.98-4.44 (m, 9H), 4.25 (m, 1H), 3.72-3.50 (m, 4H). Anal. Calc. for $C_{34}H_{36}O_6$: C, 75.53; H, 6.71. Found: C, 75.68; H, 6.80.

6.2. Phenyl 2,3,4,6-Tetra-O-benzyl-1-thio-D-glucopyrano-side (3)

To a stirred solution of **2** (108 g, 0.2 mol) and phenyl disulfide (53 g, 0.24 mol) in dichloromethane (500 mL) is added tri-n-butylphosphine (60 mL, 90%, 0.22 mol). After allowing the reaction mixture to stir at room temperature for 15 h, it is poured into a solution of saturated aqueous sodium bicarbonate (600 mL) and stirred for 10 min. The organic layer is separated, washed with water (2 x 500 mL), dried (Na_2SO_4) and concentrated. The oily residue is dissolved in hexane (500 mL) and chilled to 0 °C to give compound **3** (75 g, 60%) as a white solid (mp 85-86 °C, Lit. 84-85 °C for β-thio compound; See, Ferrier, R. J. et al. *Carbohyd. Res.* (1973) 27:55). TLC (EtOAC:Hexane 1:3) Rf 0.6. IR (KBr): 3061, 3030, 2900, 2865, 1584, 1494, 1453, 1358, 1125, 1085, 1070, 1029 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.70-7.00 (m, 25H), 4.90-4.40 (m, 9H), 3.80-3.40 (m, 6H). Anal. Calc. for $C_{40}H_{40}O_5S$: C, 75.92; H, 6.38, S, 5.06. Found: C, 75.99; H, 6.39; S, 5.12.

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6.3 Phenyl 2,3,4,6-Tetra-O-benzyl-1-thio-D-glucopyranoside S-Oxide (4)

To a stirred cooled (-78 °C) solution of 3 (130 g, 0.2 mol) in dichloromethane (400 mL) is added dropwise over a period of 20 min a solution of mCPBA (74%, 58.31 g, 0.25 mol) in dichloromethane (300 mL). The mixture is stirred and allowed to warm up to -30 °C. The mixture is then filtered. The filtrate is washed with saturated aqueous sodium bisulfite (2 x 300 mL), sodium bicarbonate (2 x 400 mL), brine (400 mL) and water (2 x 400 mL). The organic layer is dried (Na_2SO_4) and concentrated. Flash chromatography (CH_2Cl_2:EtOAC 9:1) of the residue furnishes sulfoxide mixture 4 (127 g, 95%) as a white solid (mp 120-122 °C). TLC (EtOH:CH_2Cl_2 1: 9) Rf 0.3. IR (KBr): 3060, 3030, 2910, 2867, 1495, 1450, 1360, 1210, 1136, 1092, 1049 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.72-7.14 (m, 25H), 5.12-4.42 (m, 9H), 4.40-3.30 (m, 6H). Anal. Calc. for $C_{40}H_{40}O_6S$: C, 74.04; H, 6.22; S, 4.93. Found: C, 74.10; H, 6.26; S, 4.99.

6.4. Methyl 3α-p-Methoxybenzoate-5β-cholan-24-oic Acid Ester(5)

A solution of methyl cholate (42.2 g, 0.1 mol), *p*-anisoyl chloride (20 mL, 0.133 mol) and DMAP (1 g) in pyridine (500 mL) is stirred and refluxed for 8 h. Additional *p*-anisoyl chloride (10 mL, 0.67 mol) is added and stirred 12 h. The reaction mixture is concentrated, and the residue is dissolved in dichloromethane (600 mL). The solution is washed consecutively with 1N HCl (2 x 500 mL) and water (3 x 500 mL), dried (Na_2SO_4) and the solvent allowed to evaporate. Crystallization of the residue from EtOAC/hexane (1:1) furnishes 5 (40 g, 72%) as a white solid (mp 179-180 °C). TLC (EtOAC:Hex-ane 7:3) Rf 0.7.

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6.5. Methyl 3α-*p*-Methoxybenzoate-7α,12α-di(2',3',4',6'-tetra-*O*-benzyl-1'α-glucosyl)-5β-cholan-24-oic Acid Ester (6)

Triflic anhydride (30 mL, 0.178 mol) is added to cooled toluene (300 mL, -78 °C) and stirred for 5 min. To this solution, a dried (by azeotropic distillation from toluene) sulfoxide **4** (97 g, 0.1495 mol) dissolved in toluene (300 mL) is added dropwise. After 15 min of stirring, a solution of dried (by azeotropic distillation with toluene) 2,6-di-ter-butyl-4-methyl-pyridine (30.8 g, 0.150 mol) in toluene (100 mL) is added to the reaction mixture and stirred for 10 min at -78 °C. To this reaction mixture, dried (by azeotropic distillation with toluene) methyl cholate derivative **5** (33.36 g, 0.06 mol) in CH_2Cl_2 and toluene (1:1, 200 mL) is added dropwise. The reaction progress is monitored by TLC. The temperature of the reaction mixture is slowly brought to -50°C (during 45 min) and during this time the spot of **5** on the TLC disappears completely. The reaction mixture is poured into a saturated aqueous solution of sodium bicarbonate (1000 mL) and stirred for 10 min. The organic layer is separated, and the aqueous layer is extracted with dichloromethane (2 x 100 mL). The combined organic layers is washed with water (3 x 500 mL), dried (Na_2SO_4) and concentrated. The residue purified by flash chromatography (EtOAC:Hexane = 1:9 to 1:4) to furnish **6** (84 g, 87%) as a white foam (mp 46-48 °C). TLC (EtOAC:Hexane 1:3) Rf 0.3. IR (KBr): 3084, 3062, 3028, 2936, 2867, 1735, 1707, 1605, 1496, 1453, 1360, 1321, 1275, 1254, 1210, 1165, 1097, 1073, 1030 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.60-6.70 (m, 43H), 5.95 (d, 1H, J = 9Hz), 4.99 (d, 1H, J = 3.6Hz), 4.93 (d, 1H, J = 6Hz), 4.88-3.29 (m, 31H), 2.68-0.65 (m, 37H). Fab MS: 1624 $(M+Na)^+$. Anal. Calc. for $C_{101}H_{116}O_{17}$: C, 75.71; H, 7.30. Found, C, 75.59; H, 7.31.

6.6. 7α,12α-Di(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic Acid (7)

To a stirred solution of 6 (24 g, 15 mmol) in THF (150 mL), NaOH (10 g, 250 mmol) in 95% Ethanol (200 mL) is added and refluxed for 48 h, as shown in Fig. 2. The reaction mixture is then concentrated, and the residue is dissolved in ethyl acetate (300 mL), washed with water (2 x 250 mL), saturated aqueous sodium bicarbonate (2 x 300 mL), brine (300 mL) and dried (Na_2SO_4). Solvent is evaporated and the resulting compound 7 (18.5 g, 85%) is used for the next step without further purification. TLC (EtOAC:Hexane 1:3) Rf 0.4.

6.7. Methyl 7α,12α-Di(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic Acid Ester (8)

A cooled (-10 °C) solution of diazomethane in ether (100 mL, generated from 5.35 g of diazalid, 25 mmol) is added to a cooled (-10 °C) solution of 7 (18.5 g, 12.74 mmol) in ether (100 mL). After 1 h, excess diazomethane is destroyed by adding glacial acetic acid (2 mL). The reaction mixture is washed consecutively with saturated aqueous sodium bicarbonate (2 x 400 mL), brine (300 mL), and water (300 mL), dried (Na_2SO_4), and concentrated. The residue is purified by flash chromatography (EtOAC:Hexane 3:17) to furnish 8 (13 g, 70%) as a gum. TLC (EtOAC:Hexane 1:3) Rf 0.6. IR (Neat): 3450, 2925, 2866, 1736, 1453, 1362, 1158, 1071, 1030 cm^{-1}. ^1H NMR ($CDCl_3$): 7.40-6.50 (m, 40H), 5.10-3.40 (m, 33H), 2.40-0.71 (m, 38H). Anal. Calc. for $C_{93}H_{110}O_{15}$: C, 76.08; H, 7.56. Found : C, 74.79; H, 7.50.

6.8. Methyl 3β-Azido-7α,12α-di(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oicAcidEster(9)

To a cooled (0 °C) solution of methyl cholate derivative 8 (13 g, 8.87 mmol) and pyridine (2.5 mL, 31 mmol) in dichloromethane (50 mL), triflic anhydride is

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added and allowed to stir for 20 min. To this mixture, a solution of sodium azide (2.6 g, 40 mmol) in DMF/DMPU (1:1, 250 mL) is then added at -20 °C. The reaction mixture is allowed to warm up to room temperature, where it is stirred overnight. The solvents are evaporated, and the residue is dissolved in dichloromethane (200 mL), washed with water (3 x 200 mL), dried (Na_2SO_4), and concentrated. Flash Chromatography of the residue on silica (EtOAC:Hexane 3:17) furnishes 10g (75%) of **9** as a white solid (mp 112-114 °C). TLC (EtOAC:Hexane 1:4) Rf 0.6. IR (KBr): 3085, 3061, 3029, 2921, 2867, 2097, 1735, 1603, 1495, 1452, 1360, 1256, 1207, 1160, 1091, 1071, 1031 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.37-6.84 (m, 40H), 5.15 (d, 1H, J = 4Hz), 4.95 (d, 1H, J = 4Hz), 4.86-4.26 (m, 15H), 4.08-3.40 (m, 16H), 2.60-0.71 (m, 37H). Fab MS: 1515 $(M+Na)^+$. Anal. Calc. for $C_{93}H_{110}O_{14}N_3$: C, 74.76; H, 7.43; N, 2.81. Found: C, 74.84; H, 7.40; N, 2.79.

6.9. Methyl 3β-Amino-7α,12α-di(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic Acid Ester (10)

A solution of compound **9** (11 g, 7.38 mmol) and Ph_3P (5.76 g, 22 mmol) in 90% aqueous THF (100 mL) is stirred and refluxed for 48 h. The reaction mixture is concentrated, and the residue is purified by flash chromatograph (CH_2Cl_2 and then CH_2Cl_2:EtOH = 98:2 to 9:1) to give the 3-amino compound **10** (6 g, 56%) as a white solid (mp 43-45 °C). TLC (EtOH:CH_2Cl_2 1:19) Rf 0.15. IR (KBr): 3418, 2922, 2868, 1736, 1496, 1453, 1362, 1161, 1071, 1032 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.38-6.84 (m, 40H), 5.10-3.48 (m, 33H), 2.62-0.70 (m, 37H). Anal. Calc. for $C_{93}H_{112}O_{14}N$: C, 76.08; H, 7.70; N, 0.95. Found: C, 75.82; H, 7.71; N, 0.89.

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6.10. Methyl 3β-Amino-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic Acid Ester (11)

To a solution of **10** (14.65 g, 10 mmol) in toluene (50 mL) and ethanol (200 mL) is added formic acid (15 mL) and palladium hydroxide (20%) on carbon (15 g). The resulting mixture is stirred for 24 h under a hydrogen atmosphere at 40 psi. TLC indicated incomplete hydrogenolysis. Additional formic acid (4 mL) and catalyst (4 g) is then added, and the hydrogenation reaction allowed to proceed for another 24 h. The reaction mixture is then filtered through sand over a membrane filter and concentrated. The filtrate is then mixed with ethyl acetate to form a precipitate. (Some of the methanol solvent from the hydrogenation reaction may need to be removed.) The filtered precipitate is then dissolved in 25 mL deionized water and freeze-dried. Flash Chromatography gives 2.82 g (38%) of 11 as white foam (mp 170-172 °C, decomp.). TLC ($MeOH:CH_2Cl_2$:Isopropylamine 2:2:1) Rf 0.15. IR (KBr): 3450, 2932, 1736, 1595, 1451, 1381, 1151, 1023 cm-1. ^1H NMR ($CDCl_3$): δ 5.05 (d, 1H), 4.80 (d, 1H), 3.91-3.10 (m, 15H), 2.50-0.58 (m, 37H). MS (Fab): 746 $(M+H)^+$. Anal. Calc. for $C_{37}H_{63}O_{14}N$: C, 59.56; H, 8.52; N, 1.88. Found: C, 54.60; H, 8.47; N, 2.49.

The corresponding 3α-amino compound can be obtained from the 3β-hydroxy starting material similarly. The 3β-hydroxy starting material can be obtained, for example, by treatment of methyl cholate with diethyl azidodicarboxylate in the presence of formic acid and triphenyl phosphine with inversion of stereochemistry to provide the methyl 3β-O-formylcholate, which, subsequently, can be hydrolyzed or manipulated, as needed.

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6.11. Methyl 3α-p-Methoxybenzoate-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic Acid Ester

To a solution of 6 (10 mmol; see, above) in toluene (50 mL) and ethanol (200 mL) is added formic acid (15 mL) and palladium hydroxide (20%) on carbon (15 g). The resulting mixture is stirred for 24 h under a hydrogen atmosphere at 40 psi. (Additional formic acid and catalyst can be added, if desired, if TLC analysis reveals that the reaction is incomplete after the initial 24 h reaction period. A second 24 h reaction period can then be initiated.) The reaction mixture is then filtered through sand over a membrane filter and concentrated. The filtrate is then mixed with ethyl acetate to form a precipitate. (Some of the methanol solvent from the hydrogenation reaction may need to be removed.) The filtered precipitate is then dissolved in 25 mL deionized water and freeze-dried. Subjecting the residue to flash column chromatography gives the title compound in ca. 38% yield.

^{1}H NMR (CD_3OD): δ 0.71 (s, 3H, 18-H), 0.90 (d, 3H, 21-H, J = 6.6Hz), 0.93 (s, 3H, 19-H), 1.0-2.6 (m), 3.2-3.4 (m, 2H), 3.55 (s, 3H, CO_2CH_3), 365 (m), 376 (s, 3H, anisoyl-4-methyl), 4.83 (d, 1H, anomeric H), 5.02 (d, 1H, anomeric H), 6.87 (d, 2H, anisoyl aromatic, J = 9Hz), 7.92 (d, 2H, anisoyl aromatic, J = 9Hz).

6.12. Synthesis of the Activated Ester of Deoxycholate

Triethylamine (10 mL, 71.2 mmol) is added to a stirred solution of the sodium salt of deoxycholic acid (15 g, 34.7 mmol), N-hydroxysuccinimide (7.5 g, 65.2 mmol), 1-hydroxybenzotriazole hydrate (9.3 g, 68.8 mmol, HOBT) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (13.2 g, 69.3 mmol, EDC) in dichloromethane. The mixture is stirred for 12 h. The reaction mixture is then diluted with water (150 mL) and extracted twice with

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dichloromethane. The organic layers are combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to provide a solid residue. The residue is recrystallized from ethyl acetate-petroleum ether to give 5.5 g (30%) of product. Selected ^1H resonances: (270 MHz, $CDCl_3$): δ 4.00 (br s, 1H, C12), 3.6 (m, 1H, C3), 1.03 (d, 3H, C17), 0.9 and 0.68 (s, 3H each, angular methyls of steroid).

6.13. Synthesis of the Deoxycholic Acid-Spermine Conjugate

Spermine (0.3 g, 1.18 mmol) is added to a stirred solution of the activated ester of deoxycholate (0.15 g, 0.28 mmol) and triethylamine (0.1 mL, 0.71 mmol) in dichloromethane. The mixture is stirred for 0.5 h and a precipitate is observed. The solids are filtered through a buchner funnel. The filtrate is washed with water (10 mL). The organic layer is concentrated to give a residue (0.18 g). The residue is acidified with methanolic trifluoroacetic acid. The resulting solution is purified by reverse phase chromatography to give 0.14 g (80%) of the steroid-polyamine conjugate. Selected ^1H resonances: (270 MHz, CD_3OD): δ 3.98 (br s, 1H, C12), 3.55 (m, 1H, C3), 3.4 (br t, 2H, spermine methylenes next to amide linkage), 3.0 (br s, 10H, spermine methylenes except those next to amide), 1.03 (d, 3H, C17), 0.9 and 0.68 (s, 3H each, angular methyls of steroid). High resolution mass spectrometry has confirmed the proper molecular weight.

In the same fashion, other non-glycosylated amphiphatic steroidal compounds, including but not limited to cholic acid or chenodeoxycholic acid, may be conjugated to a polyamine molecule, including but not limited to ethylene diamine, diethylene triamine, spermidine, other polyalkylenepolyamines, and the like.

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6.14. 3α-Hydroxy-7α,12α-di(1'α-glucosyl)-5ß-cholan-24-oic Acid

To a stirred solution of the methylcholate product of Example 6.11, above, (15 mmol) in THF (150 mL) is added NaOH (10 g, 250 mmol) in 95% ethanol (200 mL). The reaction mixture is refluxed for 48 h. The reaction mixture is then concentrated, and the residue is dissolved in ethyl acetate (300 mL), washed with water (2 x 250 mL), saturated aqueous sodium bicarbonate (2 x 300 mL), brine (300 mL) and dried (Na_2SO_4). Solvent is evaporated to provide the glycosteroid acid product in 80% yield. Activation of the carboxylic acid group is carried out as follows.

6.15. Synthesis of the Glycosteroid-Spermine Conjugate via the Activated Acid

Triethylamine (120 μL, 0.8 mmol) is added to a stirred solution of the glycosteroid acid product of Example 6.14 (0.3 g, 0.2 mmol), N-hydroxysuccinimide (72 mg, 0.6 mmol), 1-hydroxybenzotriazole hydrate (112 mg, 0.8 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (160 mg, 0.8 mmol) in dichloromethane. The mixture is stirred for 12 h. After this time, the reaction mixture is diluted with water (50 mL) and extracted twice with dichloromethane. The organic layers are combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to provide a solid residue 0.33 g (96%) of the activated ester.

To a stirred solution of the activated ester (0.15 g, 0.089 mmol) and triethylamine (50 mL, 0.35 mmol) in dichloromethane is added spermine (0.3 g, 0.61 mmol). The mixture is stirred for 0.5 h and a precipitate is observed. The solids are filtered over a buchner funnel. The filtrate is washed with water (10 mL). The organic layer is concentrated to give a residue (0.18 g). The

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residue is acidified with methanolic trifluoroacetic acid. The resulting solution is purified by reverse phase chromatography to give 0.14 g (85%) of the glycosteroid-polyamine conjugate.

In the same fashion, other glycosylated amphiphatic steroidal compounds, including but not limited to the mono-, di-, or triglycosylated forms (as appropriate) of cholic acid, 7-deoxycholic acid, or chenodeoxycholic acid, may be conjugated to a polyamine molecule, including but not limited to ethylene diamine, diethylene triamine, spermine, spermidine, other polyalkylenepolyamines, and the like.

6.16. Deprotection of the Protected Glycosteroid-Polyamine Conjugate

A hydrogenation flask is charged with a solution of the protected glycosteroid-spermine conjugate (0.11 g, 0.06 mmol; See, above) in a mixture of methanol (20 mL) and benzene (4 mL) or THF, followed by $Pd(OH)_2$ catalyst and formic acid (1 mL) or hydrochloric acid. The reaction mixture is shaken under a hydrogen atmosphere at 50 psi for 40 h. The catalyst is filtered off with Celite®, and the solvent is removed by evaporation under reduced pressure. The product is purified over Sephadex-LH-20 gel, eluting with MeOH, to give the desired glycosteroid-spermine conjugate.

6.17. Synthesis of the 12α-(O-Glucosyl)deoxycholic Acid- Spermine Conjugate (6, See, FIG. 5)

6.17.1. 3α-O-CBZ-Deoxycholic Acid, Methyl Ester (1)

A mixture of methyldeoxycholate (25 g, 61 mmol), benzylchloroformate (17.0 g, 14 mL, 100 mmol), dimethylaminopyridine (1.22 g, 10 mmol), pyridine (30 mL) and dioxane (150 mL) are stirred at room temperature 3h, the additional amounts of the benzylchloroformate (12.0

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g, 10 mL) are added two times in 2 h to complete reaction. Total amount of the benzylchloroformate is 41.0 g (34 mL). The reaction mixture is poured into a separatory funnel, water (500 mL) and ethyl acetate (300 mL) are added. The organic layer is washed with water (500 mL x 2), dried over sodium sulfate, concentrated to give an oil. The product is purified on flash chromatography over silica gel (EA-Hexane 1:1) providing 24.0 g (73% yield) of compound 1 as a thick oil. TLC (EA:Hexane 2:5) Rf 0.65. IR(near): 3553 (OH), 2943, 2869 (CH), 1742 (C=O), 1453, 1389, 1263 (arom.), 944, 911, 789, 747, 696 cm^{-1}.. ^1H NMR ($CDCl_3$): δ 7.38 (s, 5H), 5.15 (s, 2H), 3.6 (s, 3H), 2.0-1.0 (m, 24H), 0.96 (d,3H, J=6Hz), 0.86 (s, 3H), 0.65 (s, 3H).

6.17.2. 3α-O-CBZ-12α-(Tetra-O-benzyl-O-glucosyl) deoxycholic Acid, Methyl Ester (2)

Triflic anhydride (2.08 g, 1.26 mL, 7.4 mmol) is added to dry toluene (100 mL), chilled to -75 °C with acetone-dry ice bath, then phenylsulphenyl tetra-O-benzyl-glucopyranoside (glucosulfoxide) (5.06 g, 7.4 mmol) is added dropwise, and in 10 minutes the 2,5-tert-butyl-4-methyl-pyridine, and then 3-O-CBZ-Deoxymethyl cholate 1 is added dropwise. When TLC shows the reaction is finished, it is quenched by sodium bicarbonate (saturated solution, 200 mL) at -25 to -30 °C. The organic layer is dried over sodium sulfate, concentrated in vacuum at +50 to +60 °C. The residue on flash chromatography (EA-Hexane, 20% of EA) affords 2 (1.8 g, 29%), as thick colorless oil. TLC (EA-Hexane 2:5) Rf 0.70. ^1H NMR($CDCl_3$): δ 7.3 (m, 24H), 4.4-5.0 (m, 10H), 3.6 (s, 3H), 3.4-4.0 (m, 7H), 1.0-1.95 (m, 40H), 0.92 (d, 3H), 0.82 (s, 3H), 0.56 (s, 3H).

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6.17.3. 12α-(O-Glucosyl)deoxycholic Acid, Methyl Ester (3)

The compound **2** (1.6 g, 1.47 mmol) is dissolved in ethyl acetate (15 mL) and ethanol (50 mL) together with catalyst $Pd(OH)_2/C$ (500 mg). Using a Parr shaker, the reaction mixture is pressurized under hydrogen at 50 psi for 24 h. The catalyst is filtered off, and the filtrate is evaporated to give a crystalline residue. The residue is purified by flash chromatography (EtOH-DCM 2:8) to afford compound **3** (0.65 g, yield 72%) as white crystals, m.p. 186-188 °C. TLC (EtOH-DCM 2:8) Rf 0.5. IR (neat): 3510, 2943, 2585, 1690, 1452, 1376, 1148, 1090, 1050 cm^{-1}. ^1H NMR: δ 5.05 (d, 1H, J = 3Hz), 3.9 (s, 1H), 3.7-3.8 (m, 3H), 3.6 (s, 3H), 2.2-1.4 (m, 40H), 0.95 (d, 3H), 0.90 (s, 3H), 0.72 (s, 3H).

6.17.4. 12α-(O-Glucosyl)deoxycholic Acid, Hydrazide (4)

The methyl ester **3** (0.6 g, 1.1 mmol) is refluxed in 5 mL of EtOH-hydrazine hydrate (10:1) for 3 h. The solvent is evaporated, water (50 mL) added, then distilled off to remove excess of hydrazine hydrate. The residue is azeotroped with toluene to afford a colorless crystalline hydrazide **4** (0.50 g, yield 81%, m.p. 180-182 °C). TLC (EtOH-DCM 2:5) Rf 0.15. Anal. Calc. for $C_{30}H_{52}N_2O_8$ N 5.0. Found N 4.81. IR (KBr) 3393, 2907, 2863, 1633, 1543, 1452, 1372, 1144, 1016, 704 cm^{-1}.

6.17.5. 12α-(O-Glucosyl)deoxycholic Acid, Azide (5)

Hydrazide **4** (0.5 g, 0.88 mmol) is dissolved in 5 mL of 10% HCl at +1 to +3 °C to give a clear solution. Then $NaNO_2$ (0.14 g, 2.0 mmol) in 5 mL of water is added dropwise at +1 to +5 °C to the reaction mixture to afford a precipitate of the azide **5**. This azide is unstable and cannot be isolated in pure form. IR (KBr):

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3485-3290, 2928, 2866, 2270, and 2134 (CON_3), 1690, 1651, 1451, 1376, 1147, 1031 cm^{-1}. TLC (EtOH-DCM 2:5) Rf 0.35.

6.17.6. 12α-(O-Glucosyl)deoxycholic Acid-Spermine Conjugate (6)

The precipitate of azide **5** is fast filtered off through a glass filter with porosity 40-60 μm and washed with ice water (10 mL). While still wet, the precipitate of azide **5** is immediately transferred into a solution of spermine (0.5 g, 2.5 mmol) and triethylamine (0.5 mL) in 10 mL of water. The resulting mixture is stirred for 30 min, then heated up to 60 °C for 10 min, chilled to room temperature, and treated with acetic acid to a pH 4.5-5.0. The clear solution of spermine derivative **6** is purified by flash chromatography using a reverse-phase column CHP 20 in MeOH-Water. The spermine derivative **6** is eluted with a solvent gradient ranging from 50-100% of MeOH. The water-methanol fractions are combined and concentrated. The pH is adjusted to 3.5-3.0 with HCl. The clear solution is lyophilized to afford white, highly hygroscopic, crystalline spermine derivative **6** (0.37 g, yield 42% based on hydrazide **4**, 180 °C sinks, 200 °C decomposition). TLC (MeOH-DCM 2:8) Rf 0.1; (MeOH-isopropylamine-DCM 2:2:6) Rf 0.55. IR (KBr): 3450, 2943, 1690, 1452, 1376, 1148, 1091, 950 cm^{-1}. ^1H NMR (D_2O): δ 4.95 (d, 1H, J = 3Hz), 3.9 (s, 1H), 3.65 (m, 3H), 3.4 (m, 3H), 3.0 (m, 3H), 1.0-2.4 (m, 60H), 0.95 (d, 3H), 0.90 (s, 3H), 0.62 (s, 3H). Anal. Calc. for $C_{40}H_{74}N_4O_8 \cdot 3HCl \cdot 10H_2O$; C 46.7, H 8.91, N 5.45, Cl 10.2. Found: C 56.02, H 8.91, N 5.66, C 9.47. F.W. 739.5. Found: $M+Na^+$ = 763.

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6.18. Synthesis of the 7α-(O-Glucosyl)chenodeoxycholic Acid-Spermine Conjugate (6) (See, FIG. 6)

6.18.1. 3α-(O-Anisoyl)chenodeoxycholic Acid, Methyl Ester (1)

A mixture of methyl chenodeoxycholate (5.0 g, 12.3 mmol), anisoyl chloride (2.3 g, 2.0 mL, 13.5 mmol), dimethylaminopyridine (0.8 g, 6.5 mmol) in pyridine (15 mL) is heated at 100 °C for 3 h. Reaction mixture is poured into a separatory funnel, water (200 mL) and ethyl acetate (300 mL) is added. The organic layer is washed with 5% HCl (100 mL), water (200 mL), sodium bicarbonate, and dried over sodium sulfate. Sometimes a precipitate of the product appears between layers. This precipitate may be filtered off and combined with the product that is obtained after evaporation of ethyl acetate. Total amount is 5.2 g (yield 78%, m.p. 188-190 °C from EtOH). TLC (EA-Hexane 2:5) Rf 0.6. IR (KBr): 3513 (OH), 2938, 2851, 1730 ($COOCH_3$), 1712 (Anis-CO), 1607, 1579, 1509, 1451, 1279, 1165, 1100, 963, 770 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.03 (d, 2H), 7.96 (d, 2H), 4.85 (s, 1H), 3.85 (s, 3H), 3.65 (s, 3H), 2.0-1.0 (m, 24H), 0.96 (d, 3H), 0.90 (s, 3H), 0.66 (s, 3H).

6.18.2. 3α-(O-Anisoyl)-7α-(tetra-O-benzyl-O-glucosyl)chenodeoxycholic Acid, Methyl Ester (2)

Triflic anhydride (2.1 g, 1.27 mL, 7.4 mmol) is added to dry toluene (100 mL), chilled up to -72 to -75 °C with acetone-dry ice bath. Phenylsulphenyl glucoside (5.1 g, 7.4 mmol) in 20 mL of dry toluene is added dropwise, then in 10 mins the 2,6-di-tert-butyl-4-methyl-pyridine (1.52 g, 7.4 mmol) in toluene (15 mL) is added, and in 5 min the anisoyl derivate 1 (3.2 g, 5.9 mmol in 30 mL of dry toluene) is added dropwise. When TLC shows the starting material has disappeared,

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saturated solution of the sodium bicarbonate (150 mL) is poured, and the mixture is transferred into a separatory funnel. The organic layer is washed with water (20 mL), brine (50 mL), dried over sodium sulfate, and concentrated to give a thick oil. It is purified by flash chromatography (EA-Hexane); the product is eluted with 20% ethyl acetate. The product (4.0 g, yield 62%) is obtained as a thick colorless oil. TLC (EA-Hexane 2:5) Rf 0.65. IR (neat): 2950, 2870, 1690, 1745, 1610, 1450, 1275, 1160, 1050, 970, 775 cm^{-1}.

6.18.3. 3α-(Anisoyl)-7α-(O-glucosyl)chenodeoxycholic Acid, Methyl Ester (3)

The above obtained oil (4.0 g, 3.7 mmol) is dissolved in ethyl acetate (15 mL) and ethanol (75 mL), together with catalyst ($Pd(OH)_2/C$, 2.0 g). Formic acid (2.0 mL) is added to the mixture. The mixture is set up for hydrogenation in an 0.5 L Parr's apparatus at 50 psi for 24 h. The catalyst is filtered off, and the filtrate is evaporated to give a crystalline residue of 3 (1.8 g, yield 69%), m.p. 258-260° C (from EtOH), no decomposition. TLC (MeOH-DCM 1:9) Rf 0.35. IR (KBr): 3439 (OH), 2863, 1742 ($COOCH_3$), 1684 (anis. CO), 1606, 1284, 1260, 1022, 967, 773 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.9 (d, 2H, J = 6Hz), 6.8 (d, 2H, J = 6Hz), 4.95 (d, 1H, J = 3Hz), 4.75 (s, 1H), 3.80 (s, 3H), 3.58 (s, 3H), 3.3-3.5 (m, 4H), 2.0-1.1 (m, 30H), 0.92 (s, 3H), 0.88 (d, 3H), 0.62 (s, 3H).

6.18.4. 7α-(O-Glucosyl)chenodeoxycholic Acid, Hydrazide (4)

The methyl ester 3 (1.7 g, 3.0 mmol) is refluxed in mixture EtOH-hydrazide hydrate (20 mL + 6 mL) for 2 h. The crystals of hydrazide 4 (0.45 g, m.p. 238-240 °C) that form are separated from solution at room temperature and filtered off. The mother liquid is

concentrated, affording an additional amount of hydrazide **4** (0.65 g). Total yield 1.1 g (70%). TLC (MeOH-DCM, 2:8) Rf 0.05. IR(KBr): 3378 (NH, OH), 2927, 1697 (CONH), 1601, 1260, 1020, 980, 770 cm^{-1}.

6.18.5. 7α-(O-Glucosyl)Chenodeoxycholic Acid, Azide (5)

Hydrazide **4** (0.8 g, 1.4 mmol) is dissolved in 10 mL 10% HCl, chilled to +3 to +5 °C, then $NaNO_2$ (0.21 g, 3 mmol) in 5.0 mL of water is added dropwise affording a precipitate of azide **5**. This compound is unstable and cannot be isolated as a pure substance. TLC (EtOH-DCM 2:8) Rf 0.45. IR (KBr) : 3490-3300, 2930, 2850, 2260 and 2133 (CON_3), 1700, 1640, 1450, 1366, 1147, 1050 cm^{-1}.

6.18.6. 7α-(O-Glucosyl)chenodeoxycholic Acid-Spermine Conjugate (6)

The precipitate of azide 5 is fast filtered through a glass filter (porosity 40-60 μm), washed with ice water (5 mL), and while wet is immediately transferred into a solution of spermine (0.5 g, 2.5 mmol) and triethylamine (0.5 mL) in 10 mL of water. The mixture is stirred for 30 min, then is heated up to 60 °C for 10 min, then is chilled to room temperature. The pH is adjusted to 4.5-5.0 using acetic acid. The insoluble impurities are filtered off, and the clear filtrate of spermide **6** is purified by flash chromatography using a reverse-phase column CHP-20. The spermide **6** is eluted with a solvent gradient ranging from 40-100% of MeOH. The water-methanol fractions are combined, evaporated to dryness. Water (10 mL) and conc HCl (0.2 mL) is added, and the clear solution is lyophilized to afford white, highly hygroscopic, crystalline spermide **6** (0.50 g, yield 42% based on hydrazide **4**, m.p. 162-164 °C with decomp.). TLC (MeOH-i-

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PrOH-DCM 2:2:6) Rf 0.6. IR (KBr): 3447, 2934, 2865, 1652 (CONH), 1457, 1379, 1256, 1026, 772 cm^{-1}. ^1H NMR (D_2O): δ 4.85 (d, 1H, J = 3Hz), 3.5-3.8 (m, 8H), 3.5 (m, 6H), 3.1 (m, 2H), 2.9-3.0 (m, 10H), 2.1-1.0 (m, 40H), 0.796 (m, 6H), 0.551 (s, 3H). Anal. Calc. for $C_{40}H_{74}N_4O_8 \cdot 3HCl \cdot 10H_2O$. C 46.7, H9.44, N 5.45, Cl 10.37. Found C 60.8, H 8.97, N 4.60, Cl 6.09. F.W. 847.5. Mass-spectrum Fab.M-HCl+H^+ = 815. Found: 815.

6.19. 3α,7α,12α-Trihydroxy-5β-cholan-24-oic Acid, N-Oxysuccinimide (1) (See, FIG. 7)

A mixture of dry cholic acid (8.16 g, 20 mmol), dicyclohexeylcarbodimide (4.33 g, 21 mmol) and N-hydroxysuccini-mide (2.417 g, 21 mmol) is stirred in dry methylene chloride (200 mL) at room temperature for 6 h. The reaction mixture is filtered, and the filtrate concentrated. The residue is purified by flash chromatography through florosil (EtOH : CH2Cl2 = 1:19) giving 8 g (79% yield) of compound **1** as white a foam (mp 92-95 °C). TLC (EtOH:CH2Cl2 1:19) Rf 0.6. IR (KBr): 3385 (br), 2933, 2861, 2118, 1814, 1783, 1738, 1376, 1208, 1073 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.94 (s, 1H), 3.81 (s, 1H), 3.42 (m, 1H), 2.82 (br, 4H), 2.30-1.00 (m, 24H), 0.99 (d, 1H, J = 5.7Hz), 0.862 (s, 3H), 0.67 (s, 3H). Fab MS: 528 $(M+Na)^+$.

6.19.1. 3α,7α,12α-Trihydroxy-5β-cholan-24-oic Acid, N-(4,9-Diaza-12-amino-dodecyl) amide (2)

To a stirred solution of spermine (303 mg, 1.5 mmol) and triethylamine (1 mL) in anhydrous methylene chloride (20 mL), N-oxysuccinimidocholate 1 (505 mg, 1 mmol) in anhydrous methylene chloride (20 mL) is added dropwise during a 10 min period. The solution is then stirred for 3 h at room temperature. The reaction mixture is filtered and filtrate concentrated.

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The residue is purified by flash chromatography using CHP-20 reverse-phase resin (water and then 75% aqueous MeOH), affording 2 (360 mg, 52% yield) as white a foam (mp 140-145 °C). TLC (MeOH:CH_2Cl_2:isopropylamine 4.5:4.5:1) Rf 0.4. IR (KBr): 3350 (br), 2934, 2859, 1685, 1644, 1547, 1449, 1377, 1234, 1207, 1078, 1046 cm^{-1}. ^1H NMR (DMSO-d_6 and 2 drops of D_2O): δ 3.78 (s, 1H), 3.61 (s, 1H), 3.40-2.80 (m, 9H), 2.42-0.77 (m, 42H), 0.55 (s, 3H). Fab MS: 615 $(M+Na)^+$.

6.20. 3α,12α-Dihydroxy-7-deoxy-5β-cholan-24-oic Acid, N-Oxysuccinimide (3) (See, FIG. 8)

A mixture of dry deoxycholic acid (2.356 g, 6 mmol), dicyclohexeylcarbodimide (1.444 g, 7 mmol) and N-hydroxy-succinimide (0.806 g, 7 mmol) are stirred in dry methylene chloride (200 mL) at room temperature for 6 h. The reaction mixture is filtered, and the filtrate concentrated. The residue is purified by flash chromatography through florosil (EtOH:CH_2Cl_2 1:19), affording 1.764 g (60% yield) of compound 3 as white a foam (mp 75-80 °C). TLC (EtOH:CH_2Cl_2 1:9) Rf 0.5. IR (KBr): 3364 (br), 2934, 2862, 1814, 1783, 1738, 1655, 1627, 1449, 1376, 1208, 1068 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.97 (s, 1H), 3.62 (m, 1H), 2.82 (br, 4H), 2.70-0.83 (m, 30H), 0.67 (s, 3H). Fab MS: 512 $(M+Na)^+$.

6.20.1. 3α,12α-Dihydroxy-7-deoxy-5β-cholan-24--oic Acid, N-(12-Aminododecane)amide (4)

To a stirred solution of dodecan-1,12-diamine (600 mg, 3 mmol) and triethylamine (1 mL) in anhydrous methylene chloride (25 mL), N-oxysuccinimido-deoxycholate (3) (980 mg, 2 mmol) in anhydrous methylene chloride (25 mL) is added dropwise during 10 minute period. The contents are stirred for 14 h at room temperature. The reaction mixture is filtered, and the

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filtrate concentrated. The residue is purified by flash chromatography using CHP-20 reverse-phase resin (20%, 40%, 60%, 80% aqueous MeOH and then MeOH) to give 7 (575 mg, 50% yield) as white a foam (mp 118-120 °C). TLC (MeOH:CH_2Cl_2:isopropylamine 4.5:4.5:1) Rf 0.8. IR (KBr): 3365 (br), 2928, 2857, 1654, 1647, 1534, 1449, 1376, 1044 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.97 (s, 1H), 3.62 (m, 1H), 3.21 (q, 1H, J = 6.6Hz), 2.70-1.00 (m, 48H), 0.98 (d, 1H, J = 6.0Hz), 0.90 (d, 1H), 0.67 (s, 3H). Fab MS: 622 $(M+2Na)^+$.

6.21. 3α-Hydroxy-7α,12α-Di(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic Acid, N-Oxysuccinimide (5) (See, FIG. 9)

A solution of dry 7α,12α-di-(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic acid (1.452 g, 1 mmol), N-hydroxysuccinimide (126 mg, 1.1 mmol) and DCC (226 mg, 1.1 mmol) in dry methylene chloride is stirred at room temperature for 3 h. The reaction mixture is filtered, and the filtrate concentrated. The residue is purified by flash chromatography through a column of florosil (EtOH:CH_2Cl_2 1:19) to give 1.40 g (90% yield) of compound 5 as white a foam (mp 63-65 °C). TLC (EtOH:CH_2Cl_2 1:19) Rf 0.5. IR (KBr): 3062, 3030, 2928, 2863, 2117, 1813, 1784, 1740, 1685, 1496, 1453, 1363, 1206, 1070 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.40-6.90 (m, 40H), 5.10-3.10 (m, 33H), 2.80 (br s, 4H), 2.62-0.84 (m, 30H), 0.73 (s, 3H). Fab MS: 1572 $(M+Na)^+$.

6.21.1. 3α-Hydroxy-7α,12α-Di(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic Acid, N-(4,9-Diaza-12-aminododecane) amide (6)

To a stirred solution spermine (0.808 g, 4 mmol) and triethylamine (3 mL) in dry methylene chloride (50 mL), compound 5 (5.16 g, 3.33 mmol) in

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methylene chloride (50 mL) is added and stirred for 4 h. The reaction mixture is filtered, and the filtrate is washed with water (2 x 50 mL), dried (Na_2SO_4), and concentrated. The residue is purified by flash chromatography through a column of CHP-20 reverse-phase resin (water, then methanol) to afford compound **6** (4.9 g, 85% yield) as white a foam (mp 58-60 °C). TLC (MeOH:CH_2Cl_2:isopropylamine 4.5:4.5:1) Rf 0.2. IR (KBr): 3063, 3030, 2928, 2863, 1655, 1628, 1496, 1452, 1362, 1208, 1147, 1070, 1028 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.40-6.90 (m, 40H), 6.62 (br s, 1H), 5.03-3.20 (m, 33H), 3.00-0.86 (m, 55H), 0.72 (s, 3H). Fab MS: 1659 $(M+Na)^+$. Anal. Calc. for $C_{102}H_{132}O_{14}N_4 \cdot H_2O$: C, 74.16; H, 8.19; N, 3.35. Found: C, 73.53; H, 8.24; N, 3.72.

6.21.2. 3α-Hydroxy-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic Acid, N-(4,9-Diaza-12-aminododecyl)amide (7)

To a solution of compound **6** (2.455 g, 1.5 mmol) and 1N aqueous HCl (25 mL) in THF (50 mL), 20% palladium hydroxide on carbon (2 g, Perlman's catalyst) is added. The mixture is subjected to hydrogenalysis at 50 psi for 6 h. The reaction mixture is filtered through sand and membrane filter and concentrated. The residue is dissolved in water (5 mL) and filtered. The filtrate is purified by flash chromatography through a column of CHP-20 reverse-phase column (water, followed by MeOH:Water 1:9) to give 1.078 g (70% yield) of **7** as a white foam (mp 83-85 °C). TLC (trifluoroacetic acid:water 1:9) Rf 0.35. IR (KBr): 3365 (br), 2938, 2867, 1638, 1629, 1561, 1545, 1459, 1150, 1075, 1048, 1025 cm^{-1}. ^1H NMR (D_2O): δ 5.06 (d, 1H, J = 3.6Hz), 4.85 (d, 1H, J = 3.6Hz), 3.95 (br s, 1H), 3.78-2.88 (m, 21H), 2.28-0.76 (m, 46H), 0.64 (s, 3H). Fab MS: 940 $(M+Na)^+$. Anal. Calc. for $C_{36}H_{84}O_{14}N_4 \cdot 3HCl \cdot 5H_2O$: C, 49.66; H, 8.52; N,

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5.04; Cl, 9.44. Found: C, 49.68; H, 8.60; N, 5.06; Cl, 9.65.

6.22. Preparation of Various Poly(aminoalkylene) Amides of Deoxycholic and Chenodeoxycholic Acids (See, FIG. 10)

6.22.1. 3α,12α-Dihydroxy-7-deoxy-5β-cholan-24-oic Acid, N-(3,6,9-Triaza-11-aminoundecyl)amide (1)

To a solution of tetraethylenepentamine (0.378 g, 2.5 mmol) and triethylamine (0.3 mL) in DMF (5 mL) is added dropwise over 10 min the N-oxysuccinimidodeoxycholate (1.0 g, 2 mmol) in 5 mL of DMF. The solution is stirred overnight at room temperature, poured into water (20 mL). The precipitate obtained is washed with cold water (50 mL), dissolved in 10 mL of 2% HCl, and filtered. The solution is poured over a CHP-20 reverse phase column and eluted using a 40-80% MeOH in water solvent gradient system to afford 1.1 g (72% yield) of the trihydrochloride, pentahydrate form of the title compound, as a white powder after lyophilization (m.p. 130-132 °C). TLC (MeOH:i-$PrNH_2$:DCM 2:2:6) Rf 0.6. IR (KBr): 3419, 2934, 1642 (CONH-), 1553, 1454, 1038 cm^{-1}. ^1H NMR (D_2O): δ 3.88(s, 1H), 2.9-3.3 (m, 16H), 1.2-2.4 (m, 42H), 0.88 (d, 3H), 0.78 (s, 3H), 0.55 (s, 3H). Fab MS:696 (Base·3HCl+Na^+). Anal. Calc. for $C_{32}H_{61}N_5O_3 \cdot 3HCl \cdot 5H_2O$: C 50.3; H 9.69; N 9.17; Cl 13.95. Found: C 51.5; H 9.04; N 10.1; Cl 10.9.

6.22.2. 3α,12α-Dihydroxy-7-deoxy-5β-cholan-24-oic Acid, N-(3,6,9,12-Tetraaza-14-aminotetradecyl)amide (2)

To a solution of pentaethylenehexamine (0.58 g, 2.5 mmol) and triethylamine (0.3 mL) in DMF (5 mL) is added dropwise over 10 min the N-oxysuccinimidedeoxycholate (1.0 g, 2 mmol) in 5 mL of

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DMF. The solution is stirred overnight at room temperature, then poured into water (50 mL) to give a precipitate. The liquid phase is decanted. The semi-solid precipitate is washed successively with cold 5% NaOH (10 mL x 2) and water (10 mL), dissolved in 10 mL of 10% acetic acid, and purified by flash chromatography through a CHP-20 reverse-phase column using a 40-100% MeOH in water solvent gradient system. The fractions containing product are combined, evaporated at reduced pressure, dissolved in 2% aqueous HCl solution, and lyophilized to afford 0.75 g (42% yield) of the title compound as a white powder (m.p. 140-142 °C). TLC (MeOH:i-$PrNH_2$:DCM 2:2:6) Rf 0.65. IR (KBr): 3425, 2932, 1770 (COOH), 1643 (CONH), 1552 (COO^-), 1454, 1032 cm^{-1}. ^1H NMR (D_2O): δ 3.92 (s, 1H), 2.6-3.6 (m, 20H), 1.0-1.6 (m, 30H), 0.83 (d, 3H), 0.75 (s, 3H), 0.55 (s, 3H). Fab MS: 863 ($M+H^+$). Anal. Calc. for $C_{34}H_{66}N_6O_3 \cdot 2HCl \cdot 3AcOH$: C 55.8; H 9.28; N 9.70; Cl 8.2. Found: C 59.0; H 9.40; N 8.3; Cl 6.6.

6.22.3. 3α,7α-Dihydroxy-12-deoxy-5β-cholan-24-oic Acid, N-(4,9-Diaza-12-aminododecyl) amide (3)

To a solution of spermine (0.8 g, 2 mmol) and triethylamine (0.3 mL) in 5 mL of DMF is added dropwise the N-oxysuccinimidechenodeoxycholate (1.0 g, 2 mmol) in 5 mL of DMF. The mixture is stirred overnight at room temperature, then poured into DCM (100 mL). The precipitate of the hydroxysuccinimide is filtered, and the filtrate is evaporated to give a liquid phase, which is poured into water (100 mL). The precipitate of the product is obtained. It is dissolved in MeOH (5 mL) and passed through a CHP-20 reverse-phase column. A 30% MeOH in water solvent system is used to elute the product. The solvent is removed by evaporation, and the residue is dissolved in 1 mL of trifluoroacetic acid. The resulting

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solution is diluted up to 10 mL with water, filtered, and the filtrate subsequently lyophilized to afford 0.9 g (50% yield) of a solid (m.p. 96-100 °C). The product is soluble in water. A 5% solution of the trifluoroacetate salt of the chenodeoxycholic acid-spermine conjugate is stable at room temperature over about 12-24 h, after which a precipitate of the base separates as a slurry. TLC (MeOH:i-PrNH2:DCM) Rf 0.7. IR (KBr): 3406, 2939, 2869, 1778 (COOH), 1680 (CONH-), 1553, 1458, 1196, 834, 722 cm^{-1}. ^1H NMR (D_2O): δ 3.75 (s, 1H), 3.4 (s, 1H), 2.8-3.15 (m, 12H), 2.2-1.2 (m, 39H), 0.9 (d, 3H), 0.86 (s, 3H), 0.55 (s, 3H). Fab MS: ($M+Na^+$) = 598. Anal. Calc. for $C_{34}H_{64}N_4O_3.3CF_3COOH$: C52.5; H 7.29; N 6.09. Found: C 53.5; H 7.20; N 4.95.

6.23. Preparation of 3β-Amino-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic Acid, N-(4,9-Diaza-12-aminododecyl)amide·HCl Salt, 4 (See, FIG. 11)

6.23.1. 3β-Azido-7α,12α-di(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic Acid, N-Oxysuccinimide (1)

A solution of dry 3-β-azido-7α,12α-di-(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic acid (4.443 g, 3 mmol), N-hydroxysuccinimide (406 mg, 3.5 mmol) and DCC (722 mg, 3.5 mmol) in dry methylene chloride is stirred at room temperature for 3 h. The reaction mixture is filtered, and the filtrate concentrated. The residue is purified by flash chromatography through a florosil column (EtOAc:Hexane 1:3) to give 4 g (80% yield) of compound 1 as a white foam (m.p. 64-66 °C). TLC (EtOAc:Hexane 3:7) Rf 0.3. IR (KBr): 3325, 3088, 3062, 3030, 2924, 2867, 2099, 1815, 1785, 1742, 1206, 1070 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.40-6.90 (m, 40H), 5.02 (q, 2H, J = 3.6Hz), 4.90-3.42 (m, 31H), 2.80 (br s, 4H), 2.62-0.90 (m, 30H), 0.75 (s, 3H).

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6.23.2. 3β-Azido-7α,12α-di(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic Acid, N-(4,9-Diaza-12-aminododecyl)amide (2)

To a stirred solution of spermine (0.303 g, 1.5 mmol) and triethylamine (3 mL) in dry methylene chloride (75 mL), compound **1** (1.579 g, 1 mmol) in methylene chloride (75 mL) is added and stirred for 4 h. The reaction mixture is filtered, and the filtrate is washed with water (2 x 50 mL), dried (Na_2SO_4), and concentrated. The residue is purified by flash chromatography through a CHP-20 reverse-phase resin (eluant: water and then methanol) to afford compound **2** (1.46 g, 86% yield) as a white foam (m.p. 60-62 °C). TLC ($MeOH:CH_2Cl_2$: isopropylamine 4.5:4.5:1) Rf 0.5. IR (KBr): 3432 (br), 3087, 3062, 3030, 2925, 2865, 2098, 1670, 1663, 1656, 1640, 1630, 1496, 1452, 1364, 1071, 1028 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.40-6.90 (m, 40H), 6.30-6.10 (m, 1H), 5.04-3.10 (m, 33H), 2.80-0.83 (m, 55H), 0.73 (s, 3H).

6.23.3. 3β-Amino-7α,12α-di(2',3',4',6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic Acid, N-(4,9-Diaza-12-aminododecyl)amide (3)

To a stirred mixture of **2** (0.999 g, 0.6 mmol) and Raney Ni (500 mg) in ethanol (10 mL) is added dropwise over 10 min a hydrazine hydrate (0.2 mL, 4 mmol) in ethanol (10mL). The mixture is stirred for 2 h, after which it is filtered. The filtrate is concentrated under vacuum (aspirator pump). The residue is washed with water (3 x 50 mL) and dried under vacuum to give the 3-amino compound **3** (920 mg, 94%) as a white foam (m.p. 55-57 °C). TLC ($MeOH:CH_2Cl_2$:isopropylamine 4.5:4.5:1) Rf 0.5. IR (KBr): 3415 (br), 3087, 3062, 3029, 2925, 2864, 1669, 1662, 1654, 1647, 1630, 1496, 1453, 1362, 1086, 1070, 1028 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.40-6.90 (m, 40H),

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6.30-6.10 (m, 1H), 5.00-3.00 (m, 33H), 2.80-0.78 (m, 55H), 0.66 (s, 3H).

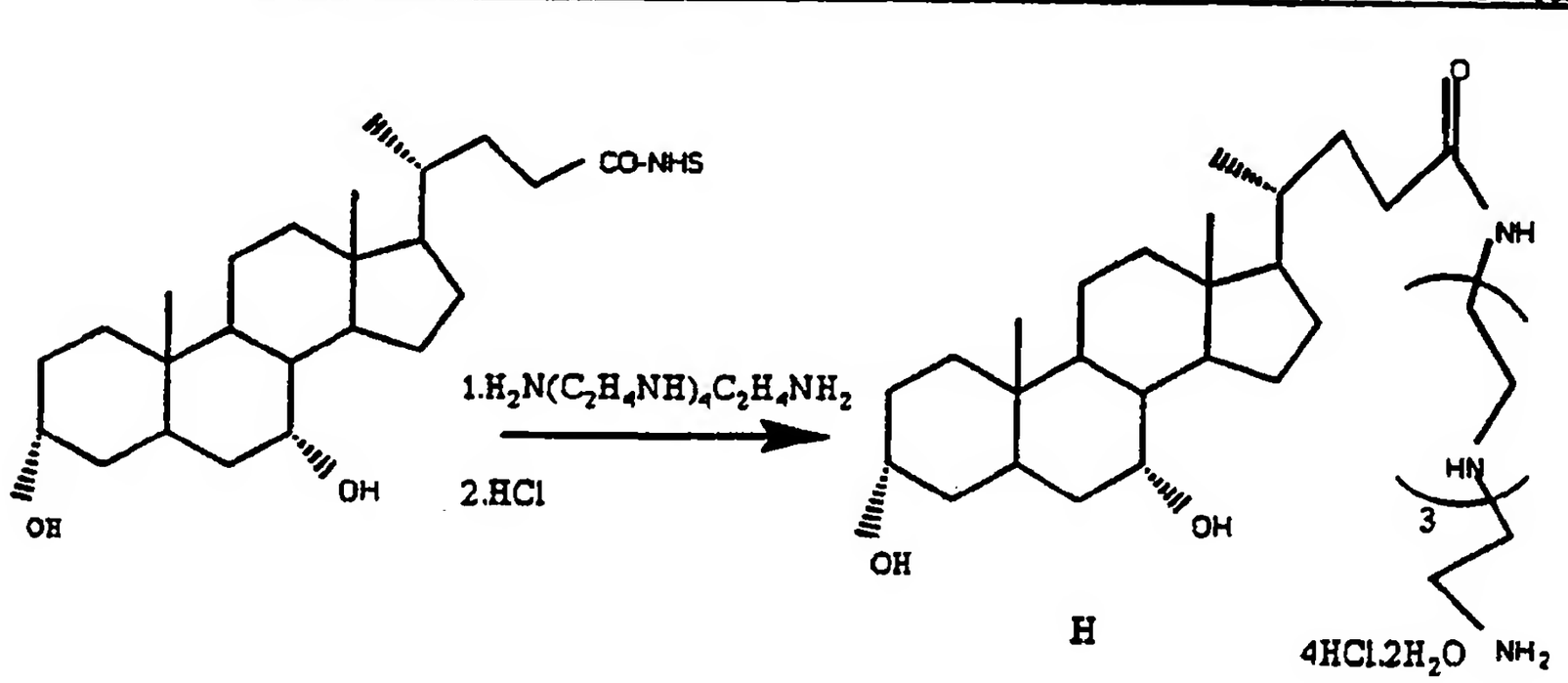
6.23.4. 3β-Amino-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic Acid, N-(4,9-Diaza-12-aminododecyl)amide·HCl Salt (4)

To a solution of compound **3** (0.91 g, 0.56 mmol) and 1N aqueous HCl (8 mL, 8 mmol) in THF (25 mL) and water (10 mL) is added 20% palladium hydroxide on carbon (0.9 g, Perlman's catalyst), and the mixture is subjected to hydrogenolysis at 50 psi for 14 h. The reaction mixture is filtered through sand and a membrane filter, then concentrated. The residue is dissolved in water (5 mL) and filtered. The filtrate is purified by flash chromatography through a CHP-20 reverse-phase column (eluant: water, followed by 2% MeOH in water) to give 260 mg (44% yield) of **4** as a white powder (m.p. 125-127 °C). TLC (trifluoroacetic acid:water 1:9) Rf 0.3. IR (KBr): 3395 (br), 2940, 1640, 1630, 1450, 1150, 1075, 1047, 1023 cm^{-1}. ^1H NMR (D_2O): δ 5.09 (br s, 1H), 4.87 (br s, 1H), 3.98 (br s, 1H), 3.78-2.88 (m, 21H), 2.60-1.00 (m, 40H), 0.91 (s, 3H), 0.82 (d, 3H, J = 5.1Hz), 0.66 (s, 3H).

Hence, the present invention also contemplates various compounds selected from non-glycosylated, monoglycosylated, and bis(glycosylated) bile acid-poly(aminoalkylene) or aminoarylene conjugates, including, in particular, 3α,12α-dihydroxy-7-deoxy-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide (deoxycholic acid-spermine conjugate); 3α-hydroxy-7α,12α-di(1'α-glucosyl)-5ß-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl) amide (bis(glycosylated)cholic acid-spermine conjugate); 3α-hydroxy-12α-(1'α-glucosyl)-7-deoxy-5ß-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide (12α-(O-glucosyl)deoxycholic acid-spermine conjugate); 3α-hydroxy-7α-(1'α-glucosyl)-12-

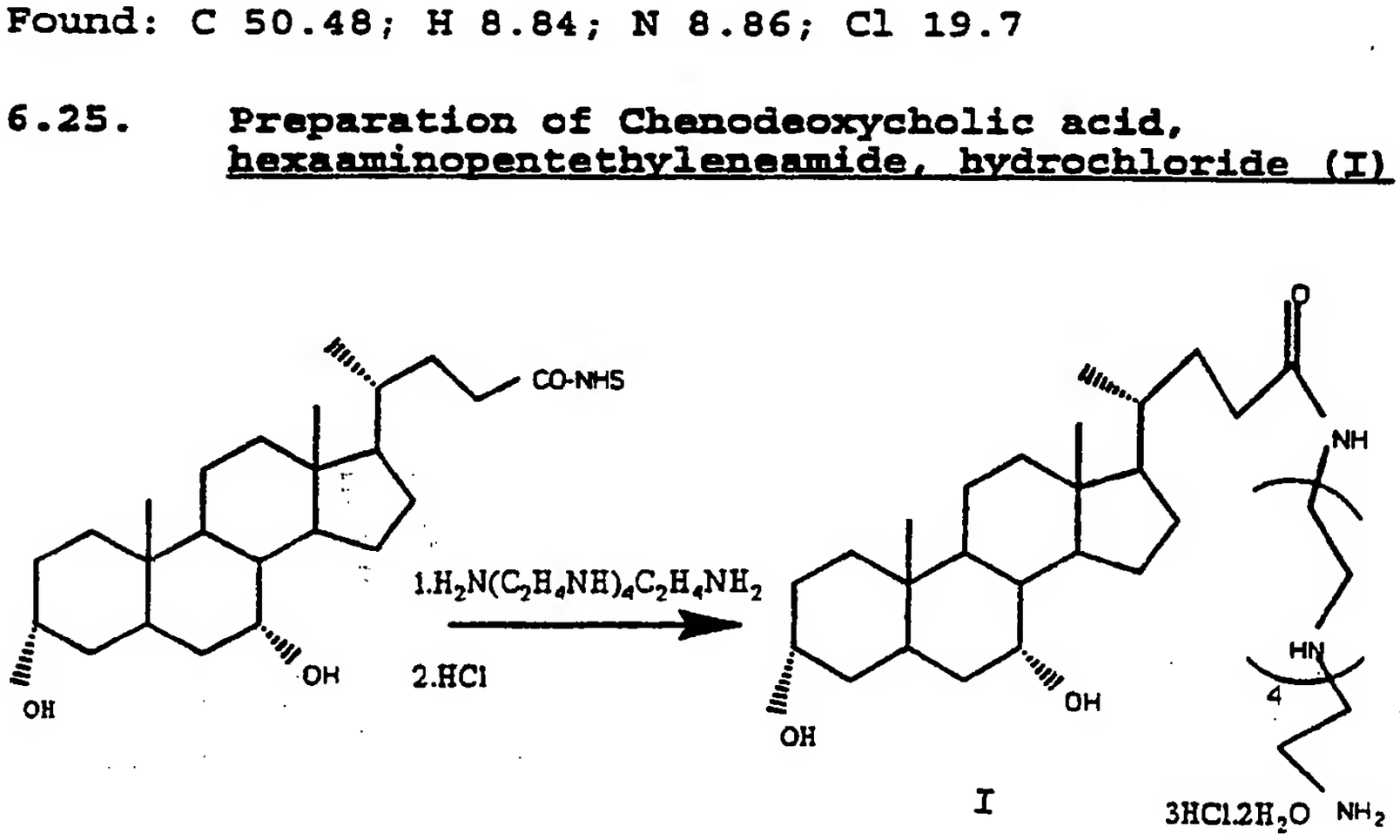
deoxy-5ß-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide (7α-(O-glucosyl)chenodeoxycholic acid-spermine conjugate); 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl) amide; 3α,12α-dihydroxy-7-deoxy-5β-cholan-24-oic acid, N-(12-aminododecane)amide; 3α-hydroxy-7α,12α-di(2',3',4',6'-tetra-*O*-benzyl-1'α-glucosyl)-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecane)amide; 3α-hydroxy-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide; 3α,12α-dihydroxy-7-deoxy-5β-cholan-24-oic acid, N-(3,6,9-triaza-11-aminoundecyl)amide; 3α,12α-dihydroxy-7-deoxy-5β-cholan-24-oic acid, N-(3,6,9,12-tetraaza-14-aminotetradecyl)amide; 3α,7α-dihydroxy-12-deoxy-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide; 3β- and 3α-amino-7α,12α-di(1'α-glucosyl)-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide; 3β- and 3α-amino-7α,12α-di(2',3',4',6'-tetra-*O*-benzyl-1'α-glucosyl)-5β-cholan-24-oic acid, N-(4,9-diaza-12-aminododecyl)amide, intermediates in their syntheses described herein, and their pharmaceutically acceptable salts.

6.24. Preparation of Chenodeoxycholic acid, pentaaminotetraethyleneamide, hydrochloride (H)



To a solution of the tetraethylenepentaamine base (1.90 g, 10 mmol) and triethylamine (1.0 g, 10 mmol) in 100 ml of DCM, N-(chenodeoxycholoyloxy)succinimide (2.46 g, 5 mmol) in DCM (dichloromethane, 50 ml) is added and the solution is stirred 48 h at R.T. The reaction mixture is diluted with 100 ml of DCM, washed with water (2 x 100 ml), dried over sodium sulfate and evaporated to dryness. The residue is dissolved in 25 ml of 10% acetic acid and filtered. The clear filtrate is purified on CHP-20 column in MeOH-water. At 40%-80% of MeOH, the product is eluted. The combined fractions are acidified by 10% HCl (5 ml). The methanol is distilled off under vacuum; the rest of the water solution is lyophilized to give 2.84 g (yield 77%, m.p. 200-203 °C decomp.) of the pentaaminotetraethyleneamide of the chenodeoxycholic acid. TLC Rf (MeOH:i-$PrNH_2$:DCM) 0.8. IR(KBr): 3350, 2974, 1665, 1635, 1551, 1539, 1460, 1470, 1377, 1077, 978, 766cm^{-1} .^{1}H NMR (D_2O):3.78(s.,1H), 2.9-3.4(m.16H), 1.8-1.2(m.39H), 0.85(d.3H), 0.76(s.,3H), 0.55(s.,3H). Fab MS:(M+H$^+$):564. MS of the chenodeoxycholic acid, pentaaminotetraethylamide is 563. Anal.for $C_{32}H_{61}N_5O_3.4HCl.2H_2O$ C 51.54; H 9.26; N 9.39; Cl 19.06; Found: C 50.48; H 8.84; N 8.86; Cl 19.7

6.25. Preparation of Chenodeoxycholic acid, <u>hexaaminopentethyleneamide, hydrochloride (I)</u>

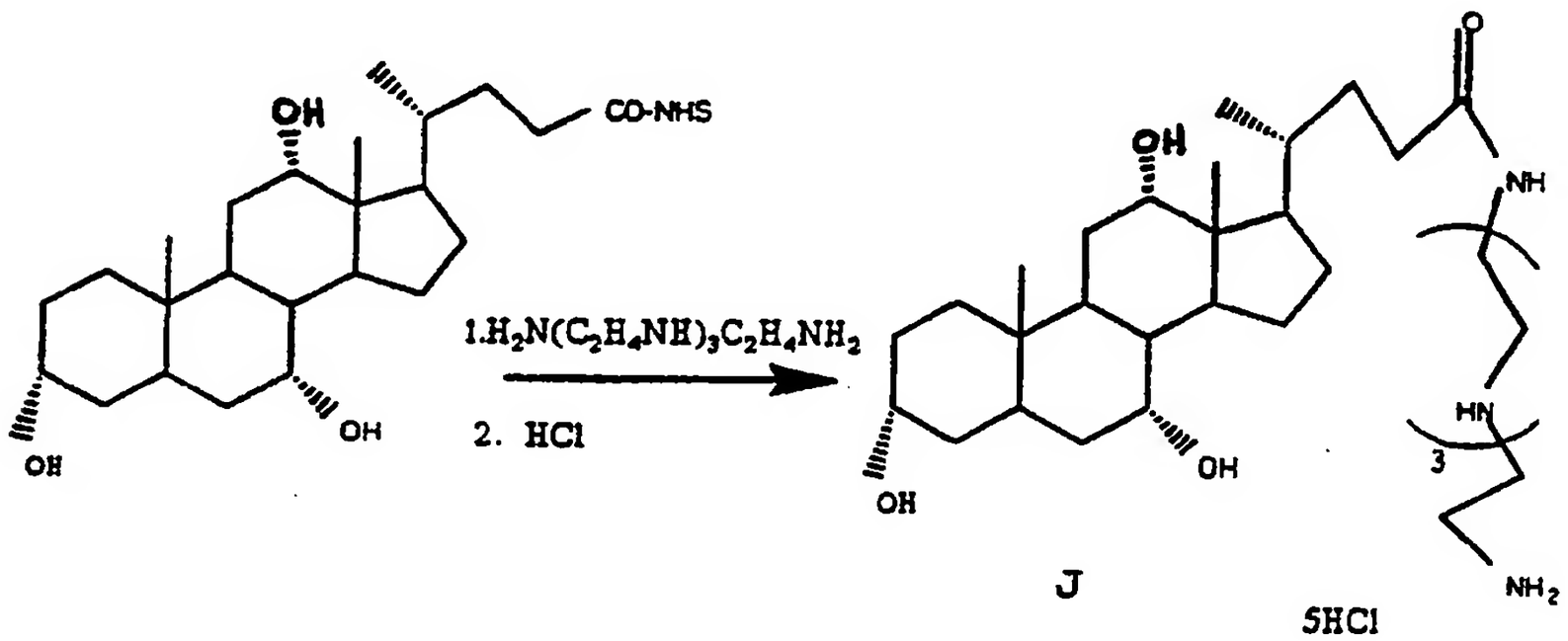


I

$3HCl.2H_2O$

To a solution of the pentaethylenehexamine (2.32 g, 10 mmol) and triethylamine (1.0 g, 10 mmol) in 50 ml of DCM the N-(chenodeoxycholoyloxy)succinimide (2.45 g, 5 mmol) in DCM (50 ml) is added. A clear solution is stirred at R.T. for 48 h. The reaction mixture is diluted with DCM (150 ml), washed with water (2 x 100 ml), dried over sodium sulfate, evaporated in vacuum to dryness, the residue is dissolved in 10% acetic acid (25ml) and is purified on CHP-20 (water-MeOH). At 30%-80% of MeOH the product is run. Combined fractions are acidified by 10% HCl and lyophilized to give 2.1 g, yield 60% of white, hygroscopic powder, m.p.168-170 °C. TLC, Rf 0.30 (MeOH:i-$PrNH_2$:DCM - 1:1:3). IR 3450, 3350,3267, 2974, 1665, 1649, 1635, 1539, 1460, 1377, 1077, 978 cm^{-1} .^1H NMR (D_2O)3.78(s.,1H), 3.2-3.5(m.20H), 1.8-1.2 (m., 28H), 0.85 (d., 3H), 0.79(s.3H), 0.55(s., 3H). Fab MS: (M+ H_2O+H$^+$) 622. Anal. for $C_{34}H_{66}N_6O_3$. 3HCl.H_2O C 55.5; H 9.66; N 11.4; Cl 14.4; Found: C 55.7; H 9.08; N 9.36; Cl 15.6.

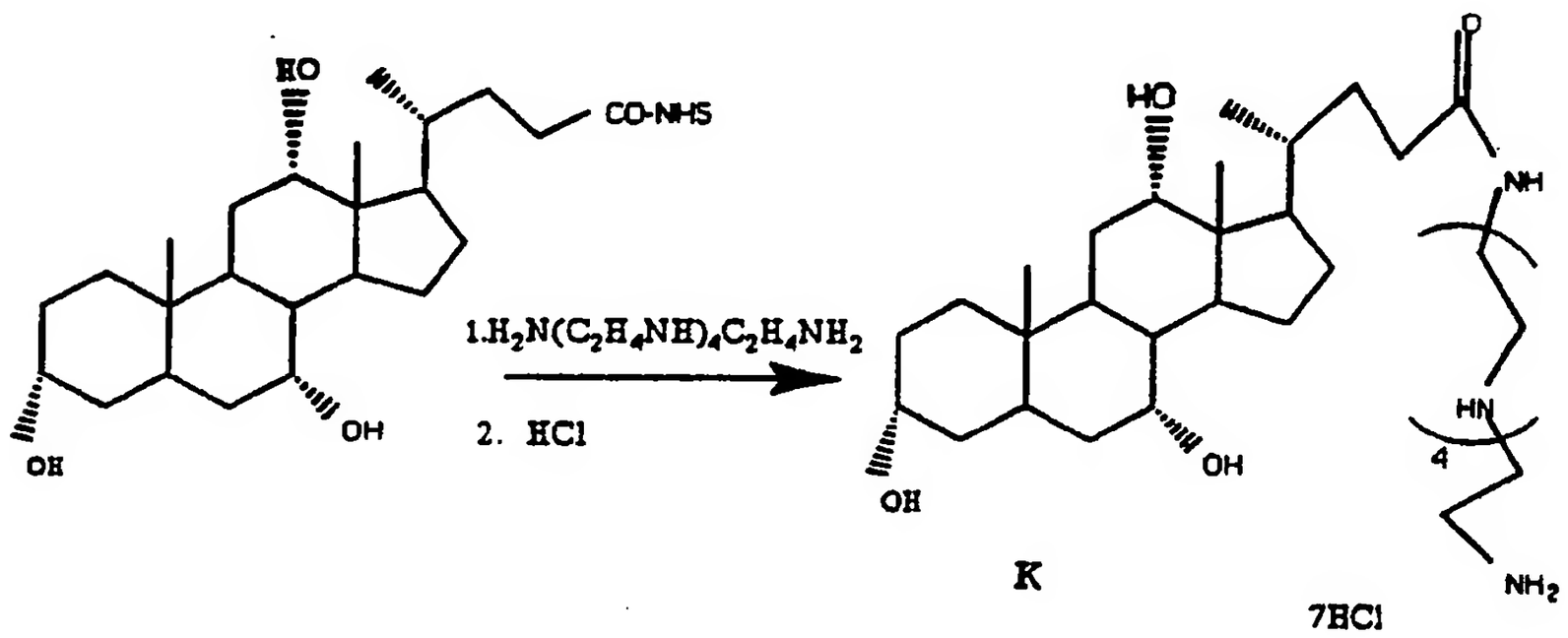
6.26. Preparation of Cholic acid, <u>pentaaminotetraethyleneamide, hydrochloride (J)</u>



To a solution of the tetraethylenepentamine (0.8 g, 5 mmol) and TEA (0.3 g, 3 mmol) in DCM (25 ml) the N-(Choloyloxy)-succinimide (1.0 g, 2.0 mmol) is added. A

clear solution is stirred at R.T. for 48 h, the reaction mixture is diluted with DCM (100 ml), washed with cold water (20 ml), dried over sodium sulfate and evaporated to dryness. The residue is dissolved in 20 ml of 5% AcOH. Purification is carried on CHP-20 reverse phase column in MeOH-water. The product elutes at 40%-80% of MeOH. The fractions of target compound are combined, methanol is distilled off, and 10 ml of 10% HCl is added. A lyophilization gives 0.70 g (yield 50%) of the pure substance, m.p.135-140 °C. TLC R_f (MeOH:i-$PrNH_2$:DCM-1:1:3) 0.8. IR(KBr): 3406, 2937, 1640(C=O), 1556, 1453, 1376, 1023 cm^{-1}. NMR ^1H (D_2O) : 3.8(s.1H), 3.65(s.1H), 3.0-3.3 (m.16H), 2.0-1.1(m.26H), 0.78(d.3H), 0.72(s.3H), 0.48(s.3H). Fab MS: ($M+H^+$)580. MS is 579. Anal. for $C_{32}H_{61}N_5O_4.5HCl$: Calc. C50.4; H 8.66; N9.18; Cl 23.29. Found. C47.27; H 8.31; N 8.57; Cl 25.63.

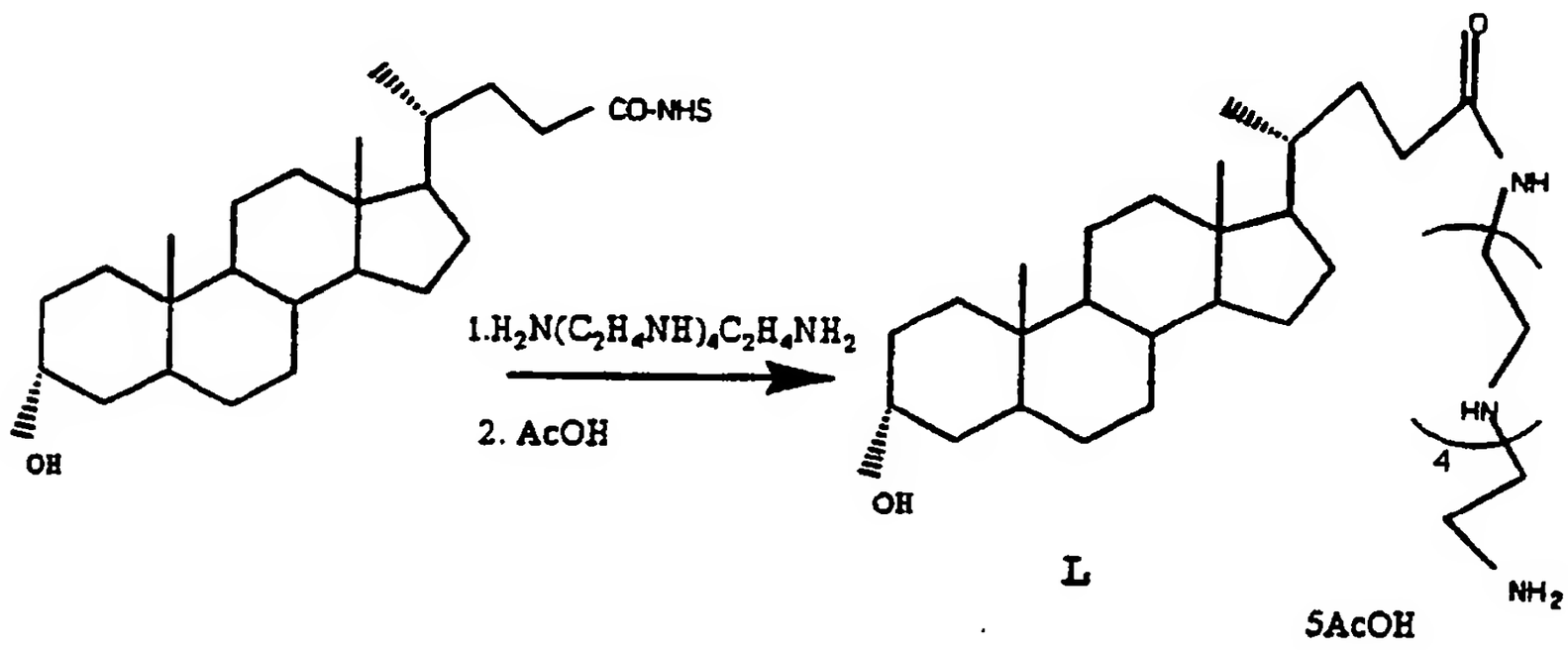
6.27. Preparation of Cholic acid, <u>hexaaminopentaethyleneamide, hydrochloride (K)</u>



To a solution of the pentaethylenehexamine (0.9 g, 5.5 mmol) and triethylamine (0.3 g, 3 mmol) in DCM (10 ml) the neat N-(Choloyloxy)succinimide (1.0 g, 2.0 mmol) is added at stirring at R.T. The reaction mixture is kept at R.T. for 48 h with stirring. At the end of this period the reaction mixture turns into a

semi-solid mixture, which is diluted with 150 ml of DCM, washed with cold water (2 x 50 ml), dried and distilled to dryness, dissolved in 10 ml of 10% AcOH, filtered from insoluble material, and purified on the reverse-phase column CHP-20 with methanol-water. The product runs at 40-70% of methanol. The combined proper fractions are distilled from methanol, 10% HCl (5 ml) is added. After lyophilization 0.96 g (55% yield) was obtained. M.P.230 °C (decomp.). TLC, Rf 0.85 (DCM:MeOH:$iPrNH_2$ - 5:1:1). IR: 3393, 2937,1646 (C=O), 1550, 1483, 1376, 1072, 1028, 774cm^{-1}. ^1H NMR (D_2O) : 3.83(s,1H), 3.67(s.1H), 3.1-3.5(m.21H), 2.0-1.4 (m.26H), 0.78(d.3H), 0.68(s.3H), 0.48(s.3H). Fab MS: ($M+H^+$) 623. Anal. Calc. for $C_{32}H_{61}N_5O_4.5HCl$: C 50.4;H 8.66; N 9.18; Cl 23.29. Found:C 47.27; H 8.31; N 8.57; Cl 25.63.

6.28. Preparation of Lithocholic acid, hexaaminopentaethyleneamide, acetate (L)



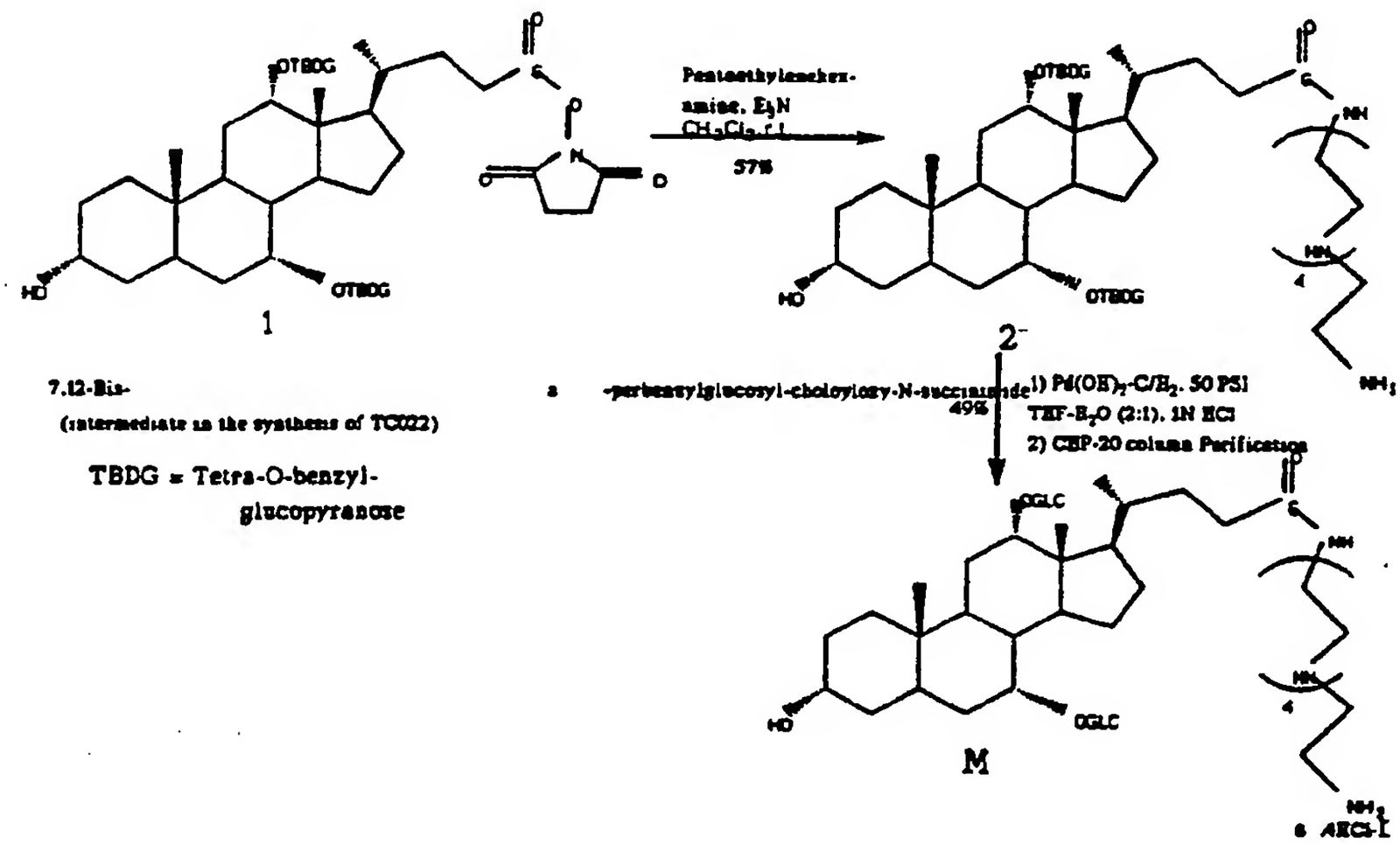
L

5AcOH

N-(Lithocholoyloxy)succinimide (1.0 g, 2.1 mmol) is added to pentaethylenehexamine (0.73 g, 3.2 mmol) and triethylamine (0.21 g, 2.1 mmol) in DCM (50 ml). The reaction mixture is kept at R.T. with stirring for 48 h., diluted with DCM (100 ml), washed with water (2 x 100 ml), and dried. The solvent is evaporated. The residue is dissolved in 50 ml of 10% AcOH during 5 h at

vigorous stirring. The cloudy solution is set up for purification on reverse phase column in MeOH-water. After a lyophilization 1.1 g (yield 60%) of the product is obtained. M.P.94 °C. TLC, Rf 0.65(DCM:MeOH:$iPrNH_2$-5:1:1). IR (KBr): 3390,2933, 2862, 1648(C=O), 1555, 1402, 1075, 656 cm^{-1}. NMR ^1H (D_2O) 3.2- 2.6 (m.19H), 1.7-1.0 (m. 29H), 0.70(s,6H), 0.42(s.3H). Fab MS:(597). Anal.for $C_{34}H_{66}N_6O_2$. 5AcOH Calc. C 59.3; H 9.66; N 9.44. Found. C 58.2; H 9.51; N 10.9.

6.29. Preparation of 3-α-Hydroxy-7α,12α-Di-(2', 3', 4', 6'-tetra-*O*-benzyl-1'α-glucosyl)-5β-cholan-24-oic acid-N-[3,6,9,12-tetra-aza-15-amino-pentadecane]-amide (2)



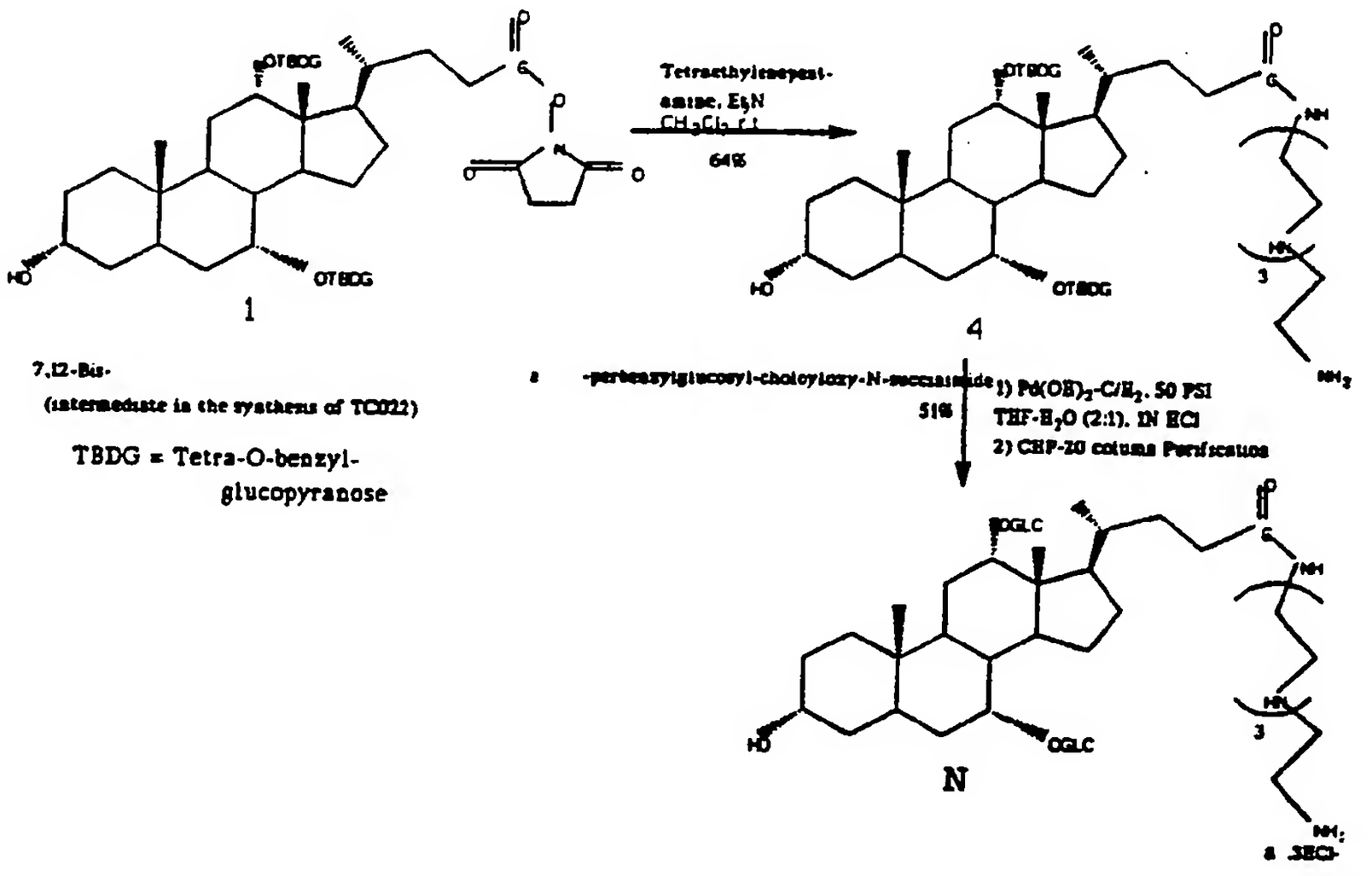
To a stirred solution of pentaethylene-hexamine (367 mg, 1.5 mmol) and triethylamine (2 mL) in dry methylenechloride (50 mL), compound 1 (1.549 g, 1 mmol) in methylenechloride (50 mL) is added dropwise and stirred for 48 h. The reaction mixture is filtered, and the filtrate is concentrated. The residue is purified on flash chromatography over CHP-20 reverse phase resin

(eluants, water and then gradually increasing to 90% methanol. Product is obtained from 90% methanol in water fractions, affording compound 2 (950 mg, 57% yield) as white foam (mp 78-80 °C). TLC R_f (solvent - MeOH:CH_2Cl_2:isopropylamine = 4:4:2) 0.1. IR (KBr): 3500 (br), 3086, 3061, 3030, 2929, 2864, 1699, 1652, 1453, 1363, 1155, 1071, 1028 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.40-6.90 (m, 40H), 5.03-3.10 (m, 33H), 2.90-0.66 (m, 65H). Fab MS: 1674 $(M+Na)^+$.

6.30. Preparation of 3-α-Hydroxy-7α,12α-Di-(1'α-glucosyl)-5β-cholan-24-oic acid-N-[3,6,9,12-tetra-aza-15-amino-pentadecane]-amide (M, above)

To a solution of compound 2 (333 mg, 0.2 mmol) and 1N aqueous HCl (3 mL, 3mmol) in THF and water (2:1, 30 mL), 20% palladium hydroxide on carbon (300 mg, Perlman's catalyst) is added and the mixture is subjected to hydrogenalysis at 50 PSI for 15 h. The reaction mixture is filtered through sand and membrane filter and concentrated. The residue is dissolved in water (5 mL) and filtered. The filtrate is purified on flash chromatography over CHP-20 reverse phase column (water, followed by MeOH:Water = 1:19, 1:4 and 2:3; product is found in 20% methanol in water fractions). The procedure provides 110 mg (49% yield) of M as a white foam (mp 180-82 °C). TLC R_f (solvent - Trifluoroacetic acid: Water = 1:9) 0.3. IR (KBr): 3394 (br), 2934, 2867, 1652, 1647, 1636, 1558, 1541, 1027 cm^{-1}. ^1H NMR (D_2O): 5.09 (d, 1H, J = 3.6Hz), 4.86 (d, 1H, J = 3.6Hz), 3.95 (brs, 1H), 3.80-2.55 (m, 15H), 2.30-0.65 (m, 56H). Fab MS:970 $(M+Na)^+$. Anal. Calc. for $C_{46}H_{86}O_{14}N_6$ .4HCl: C, 50.55 ; H, 8.30 ; N, 7.69 ; Cl, 12.97. Found: C, 50.67; H, 8.71; N, 6.70; Cl, 11.65.

6.31. Preparation of 3-α-Hydroxy-7α,12α-Di-(2', 3', 4', 6'-tetra-O-benzyl-1'α-glucosyl)-5β-cholan-24-oic acid-N-[3,6,9-tri-aza-12-amino-undecane]-amide (4)



To a stirred solution of tetraethylene-pentamine (285 mg, 1.5 mmol) and triethylamine (2 mL) in dry methylenechloride (50 mL), compound 1 (1.549 g, 1 mmol) in methylenechloride (50 mL) is added dropwise and stirred for 48 h. The reaction mixture is filtered, and the filtrate is concentrated. The residue is purified on flash chromatography over CHP-20 reverse phase resin (eluants: water and then gradually increasing to 90% methanol; product is obtained from 90% methanol in water fractions) affording compound 4 (1 g, 63.8% yield) as white foam (mp 74-76 °C). TLC R_f (solvent - MeOH:CH_2Cl_2:isopropylamine = 4:4:2) 0.1. IR (KBr): 3365 (br), 3086, 3061, 3029, 2925, 2864, 1699, 1653, 1496, 1453, 1155, 1070, 1028 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.40-6.95 (m, 40H), 5.10-3.20 (m, 33H), 2.82-0.82 (m, 57H), 0.72 (s, 3H). Fab MS: 1651 $(M+Na)^+$.

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6.32. Preparation of 3-α-Hydroxy-7α,12α-Di-(1'α-glucosyl)-5β-cholan-24-oic acid-N-[3,6,9-tri-aza-12-amino-undecane]-amide (N, above)

To a solution of compound 4 (486 mg, 0.3 mmol) and 1N aqueous HCl (4 mL, 3 mmol) in THF and water (2:1, 30 mL), 20% palladium hydroxide on carbon (400 mg, Perlman's catalyst) is added and the mixture is subjected to hydrogenolysis at 50 PSI for 15 h. The reaction mixture is filtered through sand and membrane filter and concentrated. The residue is dissolved in water (5 mL) and filtered. The filtrate is purified on flash chromatography over CHP-20 reverse phase column (water, followed by MeOH:Water = 1:19, 1:4 and 2:3; product is found in 20% methanol in water fractions) gives 160 mg (51% yield) of N as white foam (mp 151-53 °C). TLC R_f (solvent - Trifluoroacetic acid: Water = 1:9) 0.3. IR (KBr): 3390 (br), 2938, 2869, 1652, 1647, 1636, 1541, 1457, 1251, 1150, 1073, 1026 cm^{-1}. ^1H NMR (D_2O): 5.09 (br s, 1H), 4.86 (br s, 1H), 4.00 (m, 2H), 3.85-2.60 (m, 16H), 2.30-0.75 (m, 49H) and 0.66 (s, 3H). Fab MS: 927 $(M+Na)^+$. Anal. Calc. for $C_{44}H_{81}O_{14}N_5$ .3HCl: C, 52.14; H, 8.35; N, 6.91; Cl, 10.49. Found: C, 52.41; H, 8.75; N, 5.21; Cl, 9.49.

6.33. Transfection and Assay

CELLS: The day before the transfection assay COS-7 (SV40 transformed African green monkey) cells are plated at 3 X 10^4 cells/well into a 24 well multiwell dish containing DMEM + 10% FBS. Just prior to transfection the cells are washed once with serum free Opti-MEM.

TRANSFECTION ASSAY: Test Compound:DOPE (1,2-dioleoyl-sn-glycerol-3-phosphethanolamine) complex (1:1) or Lipofectin are mixed together with pSVβ plasmid DNA (5 μg/mL) at appropriate charge ratio to DNA. All solutions are made in serum free Opti-MEM. The mixtures are

incubated for 15 minutes at room temperature. The transfection mixtures are diluted to 1 μg/mL DNA with Opti-MEM and added to prewashed COS-7 cells. The COS-7 cells are incubated with the transfection mixtures for 6 hours at 37 °C. The transfections mixtures are removed and replaced with DMEM + 10% FBS. The cells are incubated for 48 hours at 37 °C then assayed for β-galactosidase activity by either X-GAL or ONPG assays.

ONPG ASSAY: COS-7 cells are washed three times with phosphate buffered saline pH 7.2 (PBS). The cells are lysed in water and frozen and thawed three times. An equal volume of O-nitrophenylpyranogalactoside (ONPG) reagent (1.76 mg/mL ONPG, 2 mM $MgCl_2$, 90 mM β-mercaptoethanol in 100 mM phosphate buffer pH 7.5) is added to either the cell extract or β-galactosidase standards. The mixture is incubated for 30 minutes at 37 °C. The absorbance at 410 nm for each mixture is determined and units of β-galactosidase activity determined from a standard curve. Cell extracts are also assayed for protein content by a Bio-Rad protein determination assay. The specific activity of the mU β-galactosidase/mg protein is determined.

X-GAL ASSAY: COS-7 cells are washed three times with PBS then fixed for 15 minutes with 2% formaldehyde + 0.2% gluteraldehyde in PBS. The cells are washed three more times with PBS and stained with X-GAL stain (5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.1% X-GAL in PBS). The plates are incubated 24 hours, and the number of stained cells is determined.

6.34 Additional Discussions

Accordingly, selected polyamine derivatives of cholic acid, deoxycholic acid, chenodeoxycholic acid, litocholic acid, and various mono and bisglycosylated derivatives thereof have been prepared (see, for example, FIGS. 12-14). The ability of these compounds to

facilitate the introduction of nucleic acids (see, e.g., FIG. 15) into cells has been explored. The results, as shown in FIG. 16, for example, suggest a direct correlation between the number of amines present in the polyamine side chain of the test compound and the observed transfection efficiency. That is, the present study has shown that transfection efficiencies increase progressively in going from a spermine side chain (four amines including the amide linkage) to a pentaamine side chain to a hexaamine side chain. The results of specific transfection experiments are provided in FIG. 16 and in Tables 6 and 7, below.

The results also suggest that each compound exhibits a maximum transfection efficiency that is dependent upon a particular compound to DNA charge ratio. For example, as shown in FIG. 17, compound E exhibits a four-fold advantage over lipofectin at a compound E to DNA charge ratio of approximately 96, whereas compound M facilitates transfection by a factor of about 12 over lipofectin at a compound M to DNA charge ratio of approximately 49.

Both the ONPG and X-GAL assays provide an effective measure of the transformation efficiencies of the compounds of the present invention, as illustrated by the results presented in FIG. 18. As noted elsewhere, the ONPG assay is a direct measure of the protein production of the transformed cells, whereas the X-GAL assay measures the density of stained cells that express heterologous β-galactosidase. A sampling of the X-GAL assay results is provided in FIG. 19.

Quite surprisingly, it has also been discovered that the transfection of COS-7 cells using a complex of compound M/DOPE is largely unaffected by the presence of serum proteins. In fact, as shown in FIG. 20, at a compound M to DNA charge ratio of about 49, increased β-galactosidase activity is observed in the presence of 1% serum relative to the β-galactosidase activity measured

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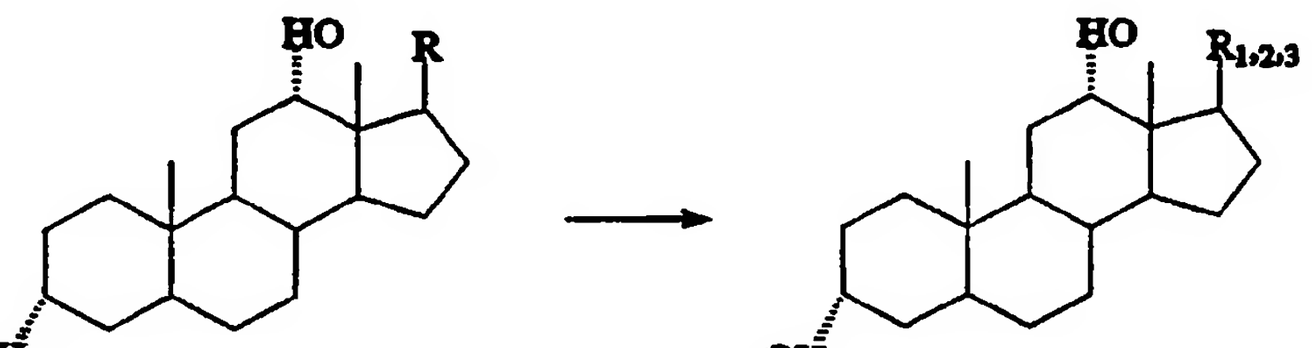
in the absence of serum. This result is noteworthy because it is known that the transfection efficiency of lipofectin is adversely affected by added serum.

Also, gel shift/DNA binding experiments show that a glycosylated cholic acid methyl ester (compound 1) and spermine alone are unable separately to retard the migration of DNA through a gel (see, FIG. 21). The results of the present invention demonstrate, quite surprisingly, the ability of a compound of the present invention, which incorporates the structural features of the glycosylated compound and spermine to retard completely the migration of DNA substantially or at a compound to DNA ratio of as low as about 10 to 1. Hence, the ability of the compounds of the present invention to bind to DNA and their consequent ability to facilitate the transformation of cells could not have been suggested by the physicochemical behavior of the individual bisglycosylated cholic acid methyl ester and spermine molecules.

6.35 Additional Compounds



R=CHCH2CH2COONSuccinyl

R_1=$CHCH_2CH_2CONH(CH_2CH_2NH)_3CH_2CH_2NH_2$ (Q)

(R) R_2=$CHCH_2CH_2CONH(CH_2CH_2NH)_4CH_2CH_2NH_2$

(S) R_3=$CHCH_2CH_2CONHCH_2CH_2CH_2NH(CH_2)_4NH(CH_2)_3NH_2$

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6.35.1 Deoxycholic acid, tetraethylenepentamide (Q)

To a solution of tetraethylenepentamine (0.378 g, 2.5 mmol) and triethylamine (0.3 ml) in DMF (5 ml) the N-oxysuccinimide-deoxycholate (1.0 g, 2 mmol) in 5 ml of DMF is added dropwise during 10 min. The solution is stirred at R.T. overnight, then poured into water (20 ml). A precipitate of the product is washed with cold water (50 ml), and dissolved in 10 ml of 2% HCl, and filtered. The solution is poured onto a CHP-20 reverse phase column, and purified in MeOH-water solvent system. The product elutes in a solvent range of 40-80% of MeOH affording 1.1 g (yield 72%, m.p. 130-132 °C) of the deoxycholic acid tetraethylenepentamide, trihydrichloride, pentahydrate, compound Q as a white powder (after lyophilization). TLC Rf (MeOH:i-$PrNH_2$; DCM-2:2:6) 0.6 IR (KBr): 3419, 2934, 1642 (CONH-), 1553, 1454, 1038 cm^{-1}. ^1H NMR (D_2O): 3.88(s., 1H), 2.9-3.3 (M. 16H), 1.2-2.4 (m.42H), 0.88 (d., 3H), 0.78 (s., 3H), 0.55 (s., 3H). Fab MS:696 (Base 3HCl+Na^+), Anal. Calc. for $C_{32}H_{61}N_5O_3$, 3HCl.$5H_2O$: C 50.3: H 9.69; N 9.17; Cl 13.95. Found: C 51.5; H 9.04; N 10.1; Cl 10.9.

6.35.2 Deoxycholic acid, pentaethylenehexamide (R)

To a solution of pentaethylenehexamine (0.58 g, 2.5 mmol) and triethylamine (0.3 ml) in DMF (5 ml) the N-oxysuccinimide-deoxycholate (1.0 g, 2 mmol) in 5 ml of DMF is added dropwise during 10 min. The resulting solution is stirred at R.T. overnight. The solution is poured in water (50 ml) to give a precipitate. The liquid phase is decanted. The semi-solid precipitate is washed with cold 5% NaOH (10 ml X 2) and water (10 ml), dissolved in 10 ml of 10% acetic acid and purified by flash chromatography on CHP-20 reverse phase column in MeOH-water solvent system. The product elutes at 40% up to 100% of MeOH. The fractions with product are combined, evaporated at reduced pressure, dissolved in 2%

HCl and lyophilized affording 0.75 g (yield 44%, m.p.) of compound **R** as a white powder, m.p. 140-142 °C. TLC Rf (MeOH;i-$PrNH_2$;DCM-2:2:6) 0.65. IR (KBr): 3425, 2932, 1770 (COOH), 1643 (CONH), 1552 (COO), 1454, 1032 cm^{-1}. ^1H NMR (D_2O):3.92(s., 1H), 2.6-3.6(m, 20H), 1.0-1.6 (M. 30H), 0.83(D., 3H), 0.75(s., 3H), 0.55(s., 3H). Fab MS: 863 ($M+H^+$).Anal. Calc. for $C_{34}H_{66}N_6O_3.2HCl.3AcOH$: C 55.8; H 9.28; N 9.70; Cl 8.2, Found: C 59.0; H 9.40; N 8.3; Cl 6.6.

6.35.3 Chenodeoxycholic acid, spermide, trifluoroacetate (S)

To a solution of spermine (0.8 g, 2 mmol) and triethylamine (0.3 ml) in 5 ml of DMF, the N-oxysuccinimide-chenodeoxycholate (1.0 g, 2 mmol) in 5 ml of DMF is added dropwise. The mixture is stirred at R.T. overnight. The solution is poured into DCM (100 ml), and the resulting precipitate of the hydroxysuccinimide is filtered; the filtrate is evaporated to give a liquid phase, which is poured into water (100 ml). The precipitate of the product is obtained. It is dissolved in MeOH (5 ml), and placed on CHP-20 reverse phase column. An MeOH-water solvent system is used. At 30% of MeOH the product elutes. The solvent is evaporated, the residue dissolved in 1 ml of trifluoroacetic acid, diluted up to 10 ml by water, filtered, and the filtrate is lyophilized affording 0.9 g (yield 50%), m.p. 96-100 °C. The product is dissolved in water to give a 5% solution of the trifluoroacetate salt of the chenodeoxycholic acid spermide. The solution is stable at R.T. about 12-24 h, then a precipitate of the base separates as a slurry. TLC Rf (MeOH:i-PrNH2:DCM) 0.7. IR (KBr): 3406, 2939, 2869, 1778 (COOH), 1680 (CONH-), 1553, 1458, 1196, 834, 722 cm^{-1}. ^1H NMR (D2O): 3.75(s., 1H), 3.4(s., 1H), 2.8-3.15(m. 12H), 2.2-1.2(m. 39H), 0.9 (d. 3H), 0.86(s. 3H), 0.55(s. 3H). Fab MS: ($M+Na^+$)=598.

Anal. Calc. for $C_{34}H_{64}N_4O_3$, $3CF_3COOH$: C52.5; H 7.29; N 6.09. Found C 53.5; H 7.20; N 4.95.

Other embodiments should be apparent to one of ordinary skill other than those specifically described above but which may, nonetheless, fall within the scope and spirit of the present invention. Those embodiments, which are specifically described, should not be construed as limiting the present invention in any way, which invention is limited solely by the following claims.

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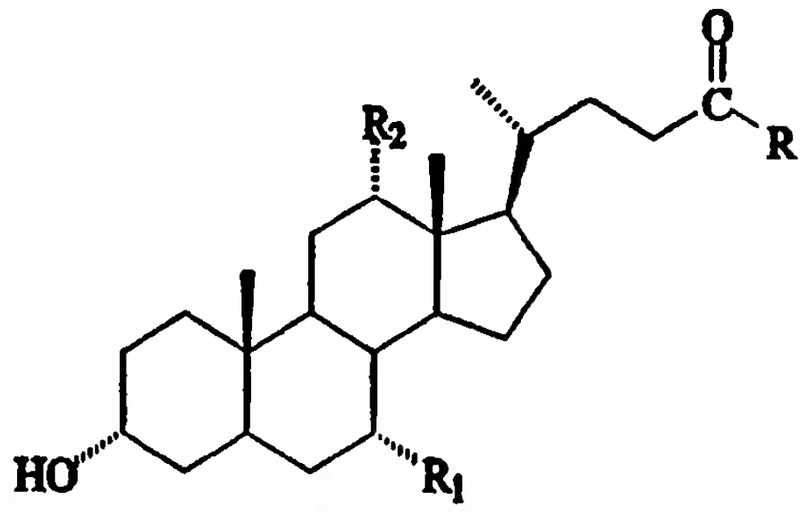
TABLE 6. SUMMARY OF TRANSFECTION RESULTS FOR C-3 OH COMPOUNDS



			Ratio (Comp/DNA)		
Comp (R)	R_1	R_2	Charge	Mole	% Transfection vs. Lipofection
Lipofectin	--	--	4(16)	7	100(100)
Spermine[a]	--	--	93	47	30
Spermine[d]	OH	OH	25	17	27
Spermine 3HCl[e]	H	O-Glc[b]	24	16	20
Spermine[f]	O-Glc	H	24	16	208
OCH_3[g]	O-Glc	O-Glc	N/A	20	12
$NH(EtNH)_3EtNH_2$[c]	O-Glc	O-Glc	96	64	127
$NH(EtNH)_3EtNH_2$ $2HCl \cdot 5H_2O$[q]	H	OH	29	15	378
$NH(EtNH)_4EtNH_2$[h]	O-Glc	O-Glc	48	20	1300
$NH(EtNH)_4EtNH_2$ $HCl \cdot 4HOAc$[r]	H	OH	15	6	827
Spermine 3TFA[i]	H	OH	5(13)	3(9)	159(460)
$NH(CH_2)_{12}NH_2$[j]	H	OH	7	20	13
Spermine 3HCl[k]	O-Glc	O-Glc	95	64	344
Spermine 2TFA[s]	OH	H	9	6	233

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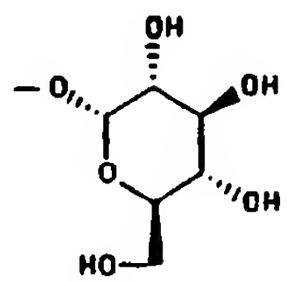
Table 6. (cont'd)

Comp (R)	R_1	R_2	Ratio (Comp/DNA) Charge	Mole	% Transfection vs. Lipofection
$NH(EtNH)_3EtNH_2$ $4HCl \cdot 2H_2O$[l]	OH	H	7	4	57
$NH(EtNH)_4EtNH_2$ $3HCl \cdot 2H_2O$[m]	OH	H	9	4	38
$NH(EtNH)_3EtNH_2$ $5HCl$[n]	OH	OH	56	28	1366
$NH(EtNH)_4EtNH_2$ $7HCl$[o]	OH	OH	30	12	1314
$NH(EtNH)_4EtNH_2$ $4HCl$	O-Glc	O-Glc	49	19	1215
$NH(EtNH)_3EtNH_2$ $3HCl$	O-Glc	O-Glc	252	126	128
$NH(EtNH)_4EtNH_2$ $5HOAc$[p]	H	H	60	28	64

[a]Spermine only.

[b]O-Glc =



[c]Et = $-CH_2CH_2-$; Comp N

[d]Comp F
[e]Comp O
[f]Comp P
[g]Comp L
[h]Comp M
[i]Comp B
[j]Comp G
[k]Comp E
[l]Comp H
[m]Comp I
[n]Comp J
[o]Comp K
[p]Comp L
[q]Comp Q
[r]Comp R
[s]Comp S

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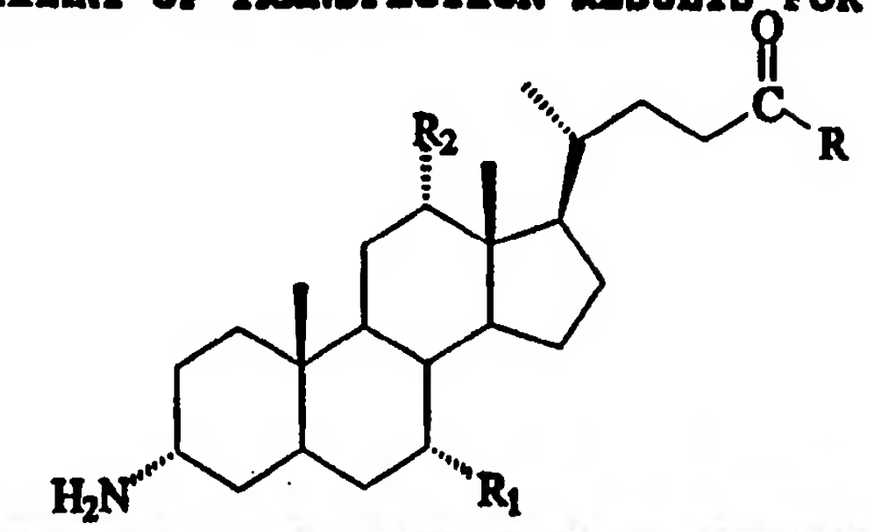
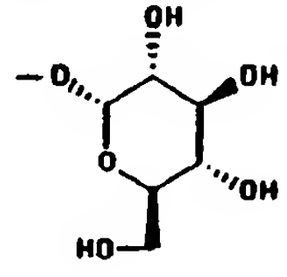
TABLE 7. SUMMARY OF TRANSFECTION RESULTS FOR C-3 NH_2 COMPOUNDS

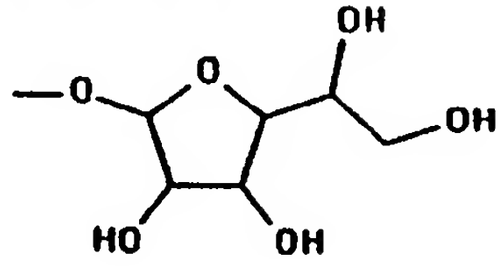


			Ratio (Comp/DNA)		
Comp (R)	R_1	R_2	Charge	Mole	% Transfection vs. Lipofection
Lipofectin	--	--	4	7	100
OCH_3	O-Glc[a]	O-Glc	10	20	2
OCH_3	O-Glc	H	4	9	213
OCH_3	H	O-Glc	32	64	64
OCH_3	O-Fur[b]	O-Fur	7	14	459
OCH_3[c]	O-Glc	O-Glc	24	49	39
Spermine	O-Glc	O-Glc	30	20	2

[a]O-Glc =



[b]O-Fur =



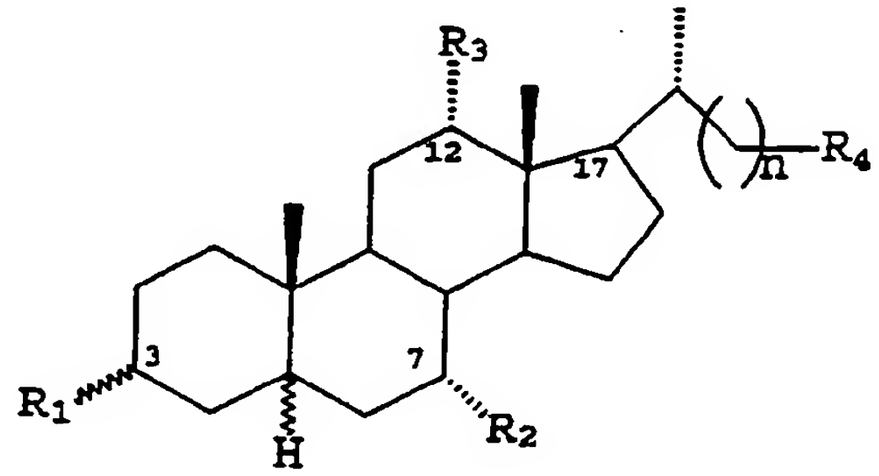
[c]This compound is the α-C-3-amino isomer.

SUBSTITUTE SHEET (RULE 26)

WHAT IS CLAIMED IS:

1. A method of introducing nucleic acid to a cell comprising:

(a) contacting a cell with the nucleic acid to be introduced in the presence of a fusogenic lipid and a compound of the formula (I):



(I)

in which

R_1 can be an H, OH, OR_5, NH_2, NHR_6 or NR_6R_7;

R_2 and R_3 may be the same or different and can be an H, OH or OR_5;

R_4 can be $CONH_2$, $CONHR_6$, $CONR_6R_7$, CH_2NH_2, CH_2NHR_6, $CH_2NR_6R_7$,
CO_2-Y-NH_2, CO_2-Y-NHR_6, or CO_2-Y-NR_6R_7;

R_5 is a protected or unprotected glycosyl moiety comprising 1-10 monosaccharide units in which the glycosidic linkage at the anomeric carbon atom of each monosaccharide unit is independently alpha or beta;

NH_2, NHR_6, and NR_6R_7 represent an unsubstituted amino group, a monosubstituted amino group, and a disubstituted amino group, respectively, in which R_6 and R_7 may be the same or different and represent a hydrocarbon group comprising 1-15 carbon atoms substituted with one or more unsubstituted, monosubstituted or disubstituted amino groups;

Y represents a linear or branched alkylene group comprising 1-10 carbon atoms;

n is an integer from 0-10;

or its acid addition or quaternary ammonium salt; and

(b) allowing said nucleic acid to remain in contact with said cell in the presence of said compound for a period of time sufficient to effect the introduction of said nucleic acid to said cell.

2. The method of claim 1 in which said introduction leads to the incorporation of said nucleic acid or at least a portion thereof within the genetic make-up of said cell.

3. The method of claim 2 in which said nucleic acid or at least a portion thereof becomes integrated within a chromosome of said cell.

4. The method of claim 2 in which said nucleic acid or at least a portion thereof is retained by said cell as extrachromosomal material.

5. The method of claim 1 in which said lipid is polar.

6. The method of claim 1 in which said lipid comprises a phospholipid.

7. The method of claim 6 in which said phospholipid contains a phosphatidylethanolamine head group.

8. The method of claim 7 in which said phospholipid is dioleoyl phosphatidylethanolamine.

9. The method of claim 7 in which said phospholipid is selected from the group consisting of palmitoyl oleoyl phosphatidylethanolamine or dimyristoyl phosphatidylethanolamine.

10. The method of claim 6 in which said phospholipid comprises a lysophospholipid.

11. The method of claim 6 in which said phospholipid is lysinyl phosphatidylethanolamine.

12. The method claim 1 in which a second lipid comprising a cationic lipid is also present in said contacting step.

13. The method of claim 12 in which said cationic lipid is selected from the group consisting of N-[1-(2,3-dioleoyloxy)pro-pyl]-N,N,N-trimethyl ammonium chloride (DOTMA), 1,2-dimyristoyl-3-trimethylammonium propane (DOTAP), and 1,2-dimyristoyl-3-dime-thylammonium propane (DODAP).

14. The method of claim 1 in which said contacting step is carried out further in the presence of diethylaminoethyldextran (DEAE).

15. The method of claim 1 in which said lipid comprises a fatty acid ester of a sugar, a glycosyl diacylglycerol, a plasmalogen, an aphingomyelin, a ganglioside, a glycerolipid, a sphingolipid or a cardiolipin.

16. The method of claim 1 in which said nucleic acid is pre-mixed with said compound before being allowed to contact said cells.

17. The method of claim 1 in which the group R_6 together with the nitrogen atom to which it is attached represents a biogenic polyamine.

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18. The method of claim 1 in which said compound can accommodate at least two positive charges.

19. The method of claim 1 in which said compound can accommodate at least three positive charges.

20. The method of claim 1 in which R_6 or R_7 represents 3-aminopropyl, 4-aminobutyl, 5-aminopentyl, N-(4-aminobutyl)-3-aminopropyl or N-[N-(3-aminopropyl)-4-aminobutyl]-3-aminopropyl.

21. The method of claim 1 in which said nucleic acid is endogenous.

22. The method of claim 1 in which said nucleic acid is exogenous.

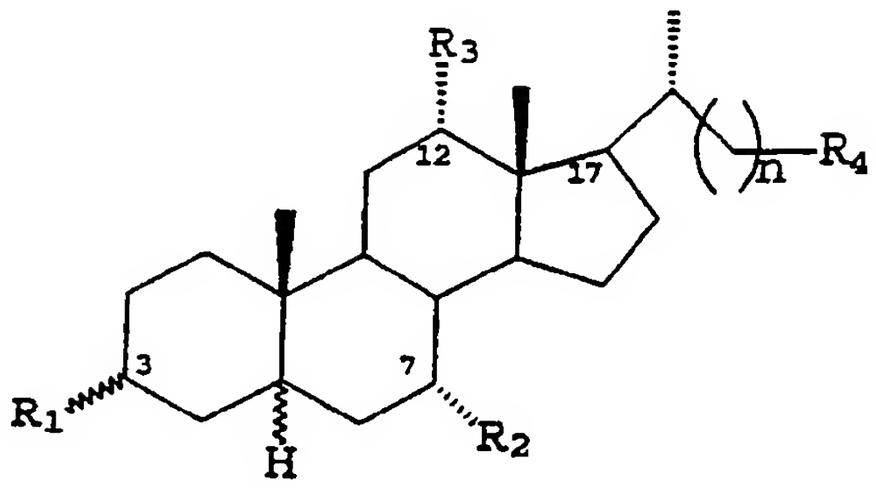
23. The method of claim 1 in which said nucleic acid comprises DNA.

24. The method of claim 1 in which said nucleic acid comprises RNA.

25. The method of claim 1 in which said nucleic acid encodes a gene.

26. The method of claim 1 in which said gene is mammalian.

27. A compound of the formula (I):



(I)

in which

R_1 can be an H, OH, OR_5, NH_2, NHR_6 or NR_6R_7;

R_2 and R_3 may be the same or different and can be an H, OH or OR_5;

R_4 can be $CONHR_6$, $CONR_6R_7$, CH_2NHR_6, $CH_2NR_6R_7$, CO_2-Y-NH_2, CO_2-Y-NHR_6, or CO_2-Y-NR_6R_7;

R_5 is a protected or unprotected glycosyl moiety comprising 1-10 monosaccharide units in which the glycosidic linkage at the anomeric carbon atom of each monosaccharide unit is independently alpha or beta;

NH_2, NHR_6, and NR_6R_7 represent an unsubstituted amino group, a monosubstituted amino group, and a disubstituted amino group, respectively, in which R_6 and R_7 may be the same or different and represent a hydrocarbon group comprising 1-15 carbon atoms substituted with one or more unsubstituted, monosubstituted or disubstituted amino groups;

Y represents a linear or branched alkylene group comprising 1-10 carbon atoms;

n is an integer from 0-3;

or its acid addition or quaternary ammonium salt; provided that said compound comprises at least one R_6 group as defined above substituted with at least one unsubstituted amino group.

28. The compound of claim 27 in which R1 has the configuration beta.

29. The compound of claim 27 in which R1 has the configuration alpha.

30. The compound of claim 27 in which at least one of R_1, R_2, and R_3 represents OH.

31. The compound of claim 27 in which at least two of R_1, R_2, and R_3 represent OH.

32. The compound of claim 27 in which all three of R_1, R_2, and R_3 represent OH.

33. The compound of claim 27 in which R_1 and R_2 represent OR_5, and R_3 represents OH.

34. The compound of claim 27 in which R_1 and R_3 represent OR_5, and R_2 represents OH.

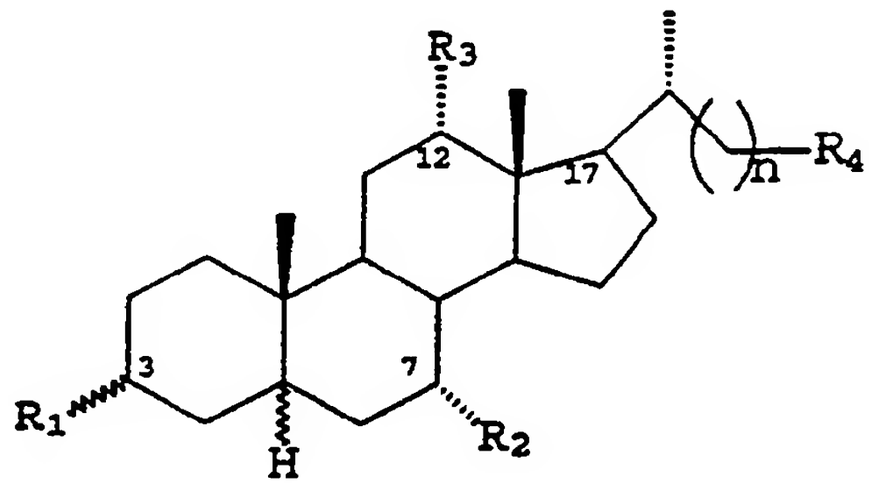
35. The compound of claim 27 in which R_2 and R_3 represent OR_5, and R_1 represents OH.

36. The compound of claim 27 in which the group R_6 together with the nitrogen atom to which it is attached represents a biogenic polyamine.

37. The compound of claim 27 which can accommodate at least three positive charges.

38. The compound of claim 27 in which R_6 or R_7 represents 3-aminopropyl, 4-aminobutyl, 5-aminopentyl, N-(4-aminobutyl)-3-aminopropyl or N-[N-(3-aminopropyl)-4-aminobutyl]-3-aminopropyl.

39. A composition for the introduction of nucleic acid to a cell comprising nucleic acid, an effective amount of a fusogenic lipid, and an effective amount of a compound of the formula (I):



(I)

in which

R_1 can be an H, OH, OR_5, NH_2, NHR_6 or NR_6R_7;

R_2 and R_3 may be the same or different and can be an H, OH or OR_5;

R_4 can be $CONH_2$, $CONHR_6$, $CONR_6R_7$, CH_2NH_2, CH_2NHR_6, $CH_2NR_6R_7$,
CO_2-Y-NH_2, CO_2-Y-NHR_6, or CO_2-Y-NR_6R_7;

R_5 is a protected or unprotected glycosyl moiety comprising 1-10 monosaccharide units in which the glycosidic linkage at the anomeric carbon atom of each monosaccharide unit is independently alpha or beta;

NH_2, NHR_6, and NR_6R_7 represent an unsubstituted amino group, a monosubstituted amino group, and a disubstituted amino group, respectively, in which R_6 and R_7 may be the same or different and represent a hydrocarbon group comprising 1-15 carbon atoms substituted with one or more unsubstituted, monosubstituted or disubstituted amino groups;

Y represents a linear or branched alkylene group comprising 1-10 carbon atoms;

n is an integer from 0-10;

or its acid addition or quaternary ammonium salt.

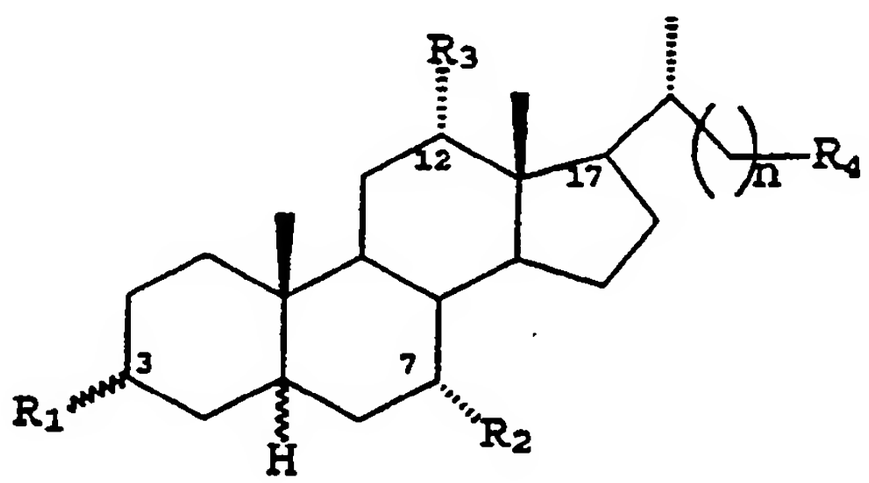
40. The composition of claim 39 in which said compound comprises at least one R_6 group as defined in claim 39 substituted with at least one unsubstituted amino group.

41. The composition of claim 40 which further comprises a liquid carrier.

42. The composition of claim 40 which further comprises a lipid.

43. The composition of claim 40 which further comprises a fusogenic lipid and a buffer.

44. A pharmaceutical composition comprising a nucleic acid and a compound of the formula (I):



(I)

in which

R_1 can be an H, OH, OR_5, NH_2, NHR_6 or NR_6R_7;

R_2 and R_3 may be the same or different and can be an H, OH or OR_5;

R_4 can be $CONH_2$, $CONHR_6$, $CONR_6R_7$, CH_2NH_2, CH_2NHR_6, $CH_2NR_6R_7$,

CO_2-Y-NH_2, CO_2-Y-NHR_6, or CO_2-Y-NR_6R_7;

R_5 is a protected or unprotected glycosyl moiety comprising 1-10 monosaccharide units in which the

glycosidic linkage at the anomeric carbon atom of each monosaccharide unit is independently alpha or beta;

NH_2, NHR_6, and NR_6R_7 represent an unsubstituted amino group, a monosubstituted amino group, and a disubstituted amino group, respectively, in which R_6 and R_7 may be the same or different and represent a hydrocarbon group comprising 1-15 carbon atoms substituted with one or more unsubstituted, monosubstituted or disubstituted amino groups;

Y represents a linear or branched alkylene group comprising 1-10 carbon atoms;

n is an integer from 0-10;

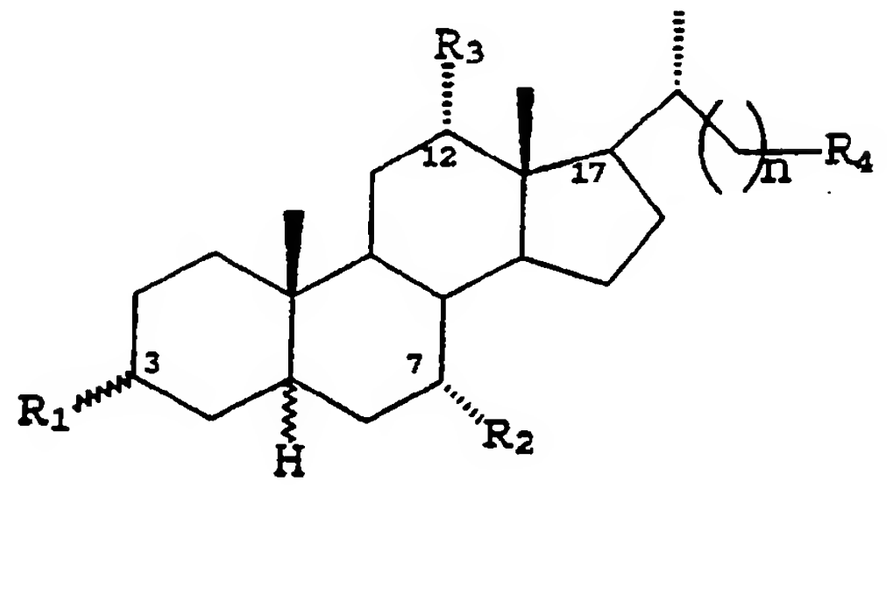
or its acid addition or quaternary ammonium salt in a pharmaceutically acceptable carrier, provided that said compound comprises at least one R_6 group as defined above.

45. The pharmaceutical composition of claim 44 in which said compound comprises at least one R_6 group substituted with at least one unsubstituted amino group.

46. The pharmaceutical composition of the claim 44 in which said nucleic acid encodes a gene.

47. The pharmaceutical composition of the claim 44 in which said nucleic acid comprises an antisense oligonucleotide sequence.

48. A method of enhancing the transformability of a host cell comprising contacting a host cell with a compound of the formula (I):



(I)

in which

R_1 can be an H, OH, OR_5, NH_2, NHR_6 or NR_6R_7;

R_2 and R_3 may be the same or different and can be an H, OH or OR_5;

R_4 can be $CONH_2$, $CONHR_6$, $CONR_6R_7$, CH_2NH_2, CH_2NHR_6, $CH_2NR_6R_7$,
CO_2-Y-NH_2, CO_2-Y-NHR_6, or CO_2-Y-NR_6R_7;

R_5 is a protected or unprotected glycosyl moiety comprising 1-10 monosaccharide units in which the glycosidic linkage at the anomeric carbon atom of each monosaccharide unit is independently alpha or beta;

NH_2, NHR_6, and NR_6R_7 represent an unsubstituted amino group, a monosubstituted amino group, and a disubstituted amino group, respectively, in which R_6 and R_7 may be the same or different and represent a hydrocarbon group comprising 1-15 carbon atoms substituted with one or more unsubstituted, monosubstituted or disubstituted amino groups;

Y represents a linear or branched alkylene group comprising 1-10 carbon atoms;

n is an integer from 0-10;

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or its acid addition or quaternary ammonium salt; provided that said compound comprises at least one R_6 group as defined above; and

(b) allowing said compound to remain in contact with said host cell while exposing said host cell to transforming nucleic acid.

49. A conjugate comprising a bile acid or its analog selected from the group consisting of cholic acid, 3-amino-5β-cholic acid, lithocholic acid, deoxycholic acid, chenodeoxy cholic acid, 3-deoxycholic acid, its salt, ester, or amide covalently linked directly or indirectly to one or more second compounds attached to C3, the C17 side chain or both, provided that said second compound or compounds do not introduce a second molecule of said bile acid or its analog into said conjugate.

50. The conjugate of claim 49 in which said 3-amino-5β-cholic acid is the 3β-amino isomer.

51. The conjugate of claim 49 in which said 3-amino-5β-cholic acid is the 3α-amino isomer.

52. A conjugate comprising a 3-amino-7α,12α-di(1'α-glucosyl)-5β-cholic acid, its salt, ester or amide covalently linked directly or indirectly to one or more second compounds attached to C3, the C17 side chain or both, provided that said second compound or compounds do not introduce a second molecule of said 3-amino-7α,12α-di(1'α-glucosyl)-5β-cholic acid, its salt, ester or amide into said conjugate.

53. The conjugate of claim 52 in which said 3-amino-7α,12α-di(1'α-glucosyl)-5β-cholic acid is the 3β-amino isomer.

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54. The conjugate of claim 52 in which said 3-amino-7α,12α-di(1'α-glucosyl)-5β-cholic acid is the 3α-amino isomer.

55. The conjugate of claim 49 in which said second compound is an amine compound.

56. The conjugate if claim 55 in which said amine compound is a polyamine compound.

57. The conjugate of claim 49 in which said polyamine compound is an alkylene diamine.

58. The conjugate of claim 49 in which said polyamine compound is a spermine.

59. The conjugate of claim 49 in which said polyamine compound is a spermidine.

60. The method of claim 1 in which said hydrocarbon group comprises a linear or branched aliphatic group.

61. The method of claim 1 in which said hydrocarbon group is cyclic.

62. The compound of claim 27 in which said hydrocarbon group comprises a linear or branched aliphatic group.

63. The compound of claim 27 in which said hydrocarbon group is cyclic.

64. The composition of claim 39 in which said hydrocarbon group comprises a linear or branched aliphatic group.

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65. The composition of claim 39 in which said hydrocarbon group is cyclic.

66. The pharmaceutical composition of claim 44 in which said hydrocarbon group comprises a linear or branched aliphatic group.

67. The pharmaceutical composition of claim 44 in which said hydrocarbon group is cyclic.

68. The method of claim 48 in which said hydrocarbon group comprises a linear or branched aliphatic group.

69. The method of claim 48 in which said hydrocarbon group is cyclic.

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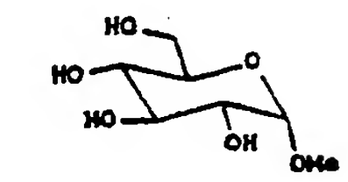
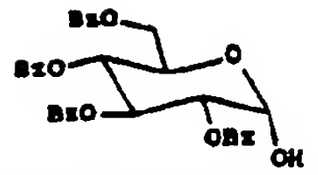


Methyl-$^{\alpha}$-D-Glucopyranoside

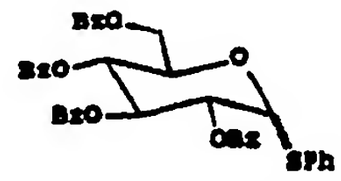
I) KOH, BzCl
II) AcOH, 3N H_2SO_4
41%



2,3,4,6-Tetra-O-benzyl$^{\beta}$-D-Glucopyranose

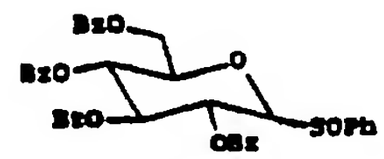
2

60% PhSSPh, Bu_3P
CH_2Cl_2



Phenyl 2,3,4,6-Tetra-O-benzyl-1-thio-D-Glucopyranoside

3

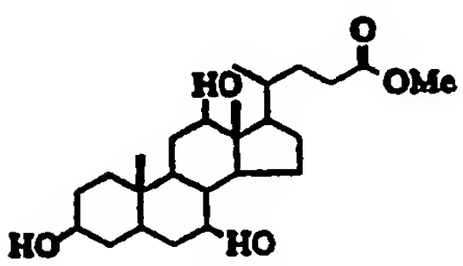


Phenyl 2,3,4,6-Tetra-O-benzyl-1-thio-D-Glucopyranose S-oxide

4

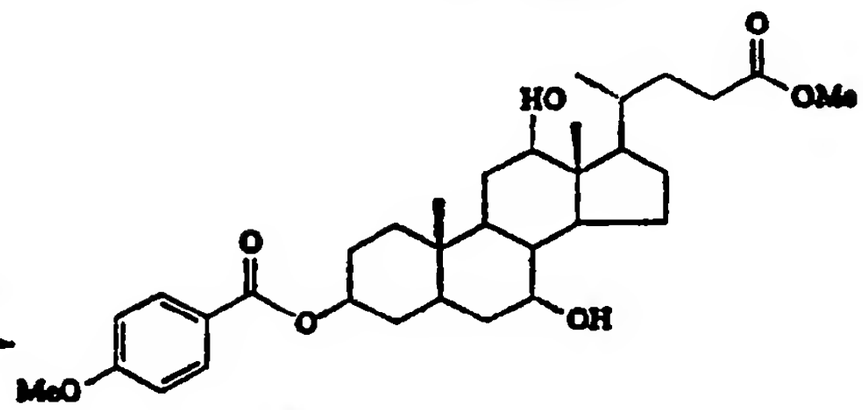
mCPBA, CH_2CH_2
78° C
95%

Bz = $PhCH_2$



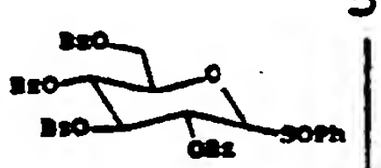
Methyl Cholate

p-Anisoyl chloride, DMAP
Pyridine
73%

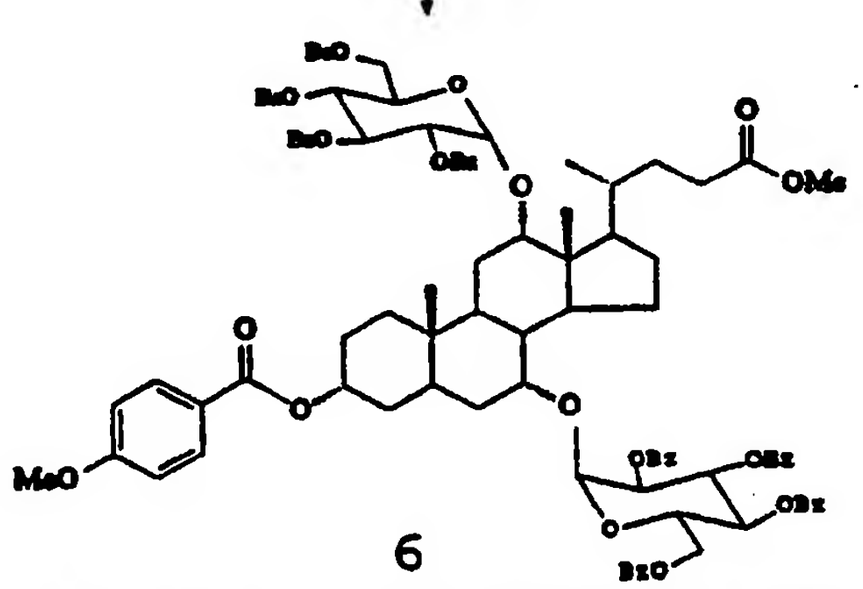


Methyl 3$^{\alpha}$-p-methoxybenzoate-5$^{\beta}$-cholate

5



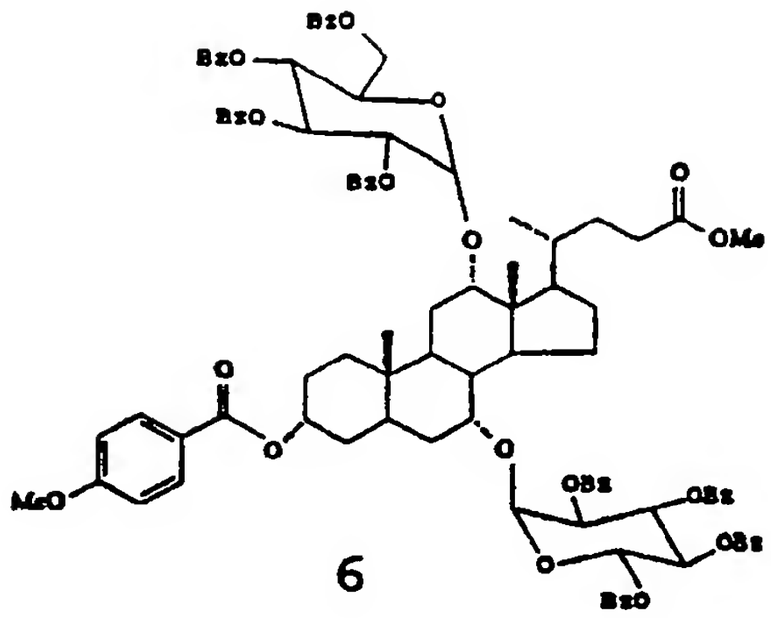
Tf_2O, Toluene,
2,6,-Di-tert-butyl-
4-methylpyridine,
-78° C
87%



Methyl 3$^{\alpha}$-p-methoxybenzoate-7$^{\alpha}$,12$^{\alpha}$-di(2,3,4,6-tetra-O-benzyl-1-$^{\alpha}$-glucosyl)-5$^{\beta}$-cholate

6

FIG. 1



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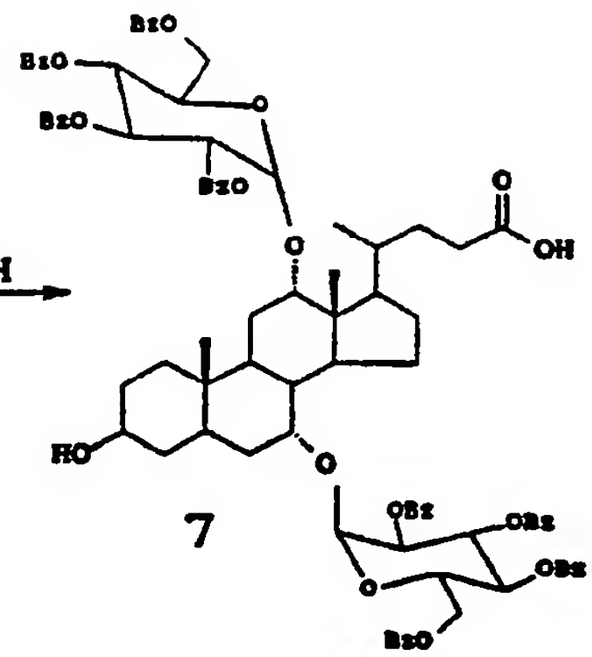
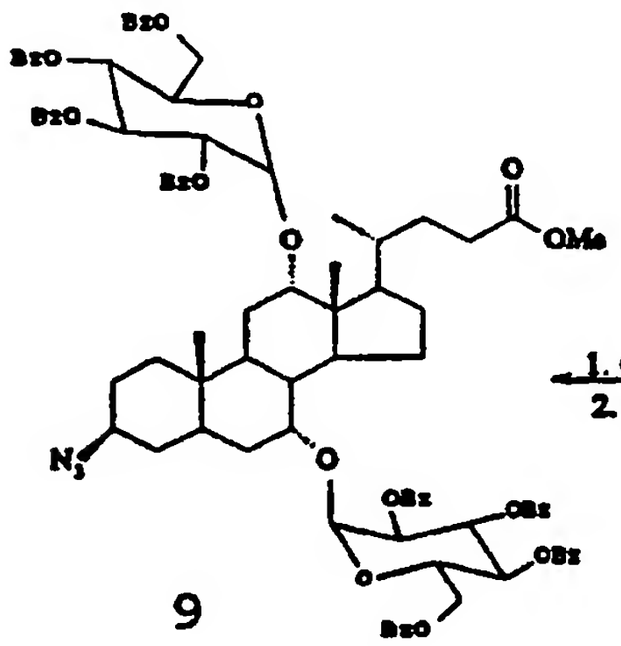


7

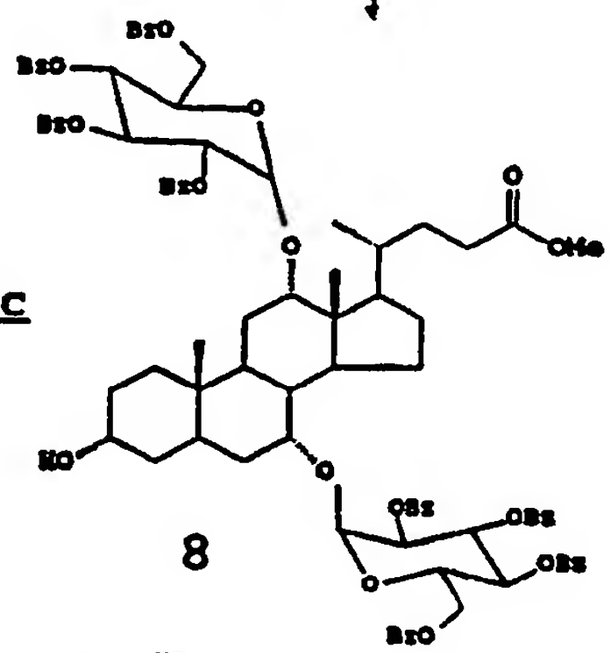
NaOH, THF, EtOH
reflux, 48 h
85%

-15°C to r.t
CH_2N_2
THF/ET_2O
70%



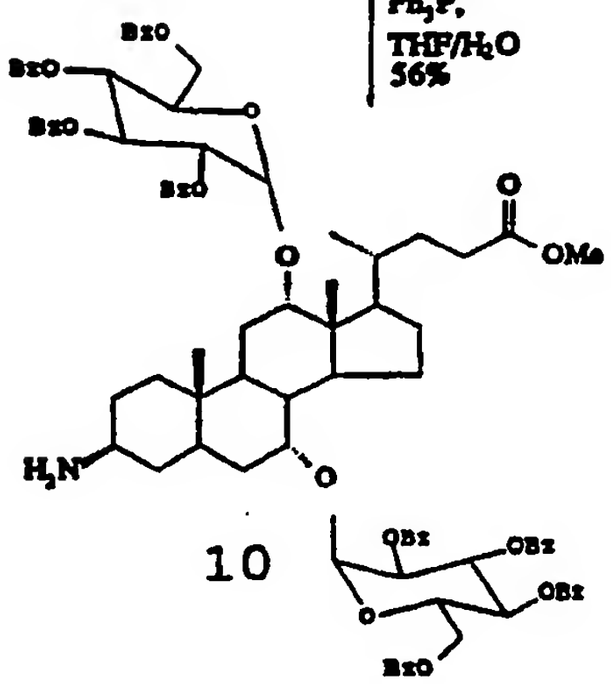
9

1. CH_3N/CH_2Cl_2, Et_2O, 0° C
2. NaN_3, DMPU/DMF
-20°C to r.t. 16 h
83%



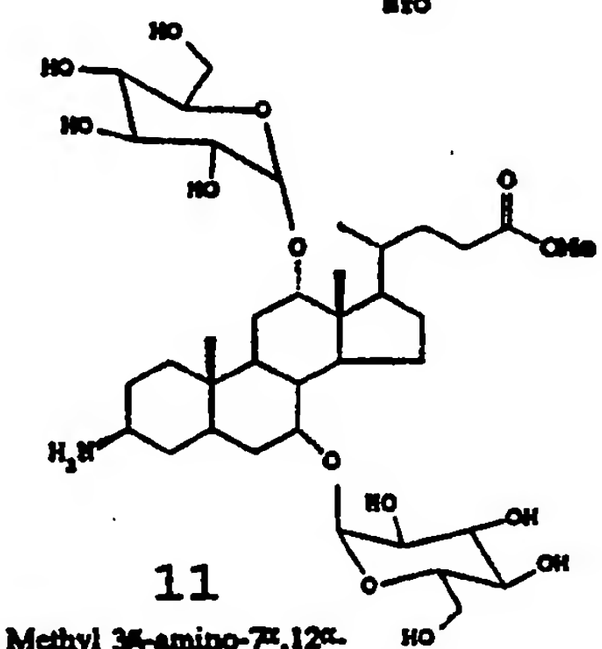
8

Ph_3P,
THF/H_2O
56%



10

$Pd(OH_2)/C$, H_2
HCO_2H, MeOH

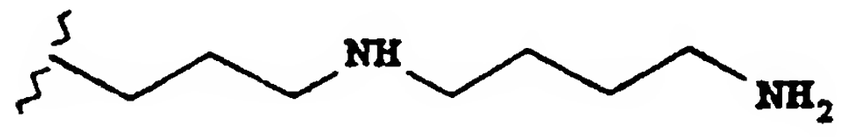


11

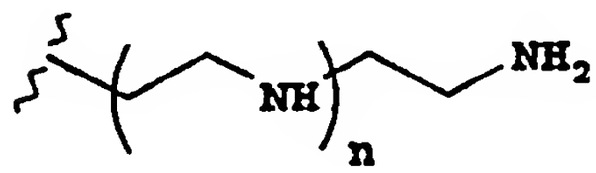
Methyl 3β-amino-7α,12α-di(1-α-glucosyl)-5β-cholate

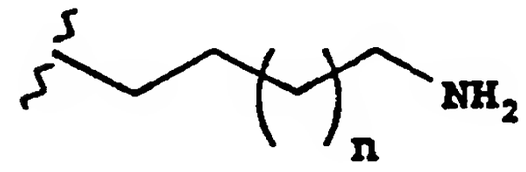
FIG. 2

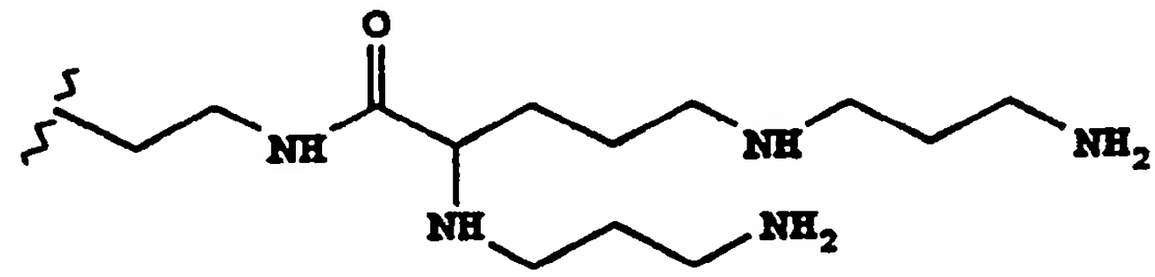
(spermine)

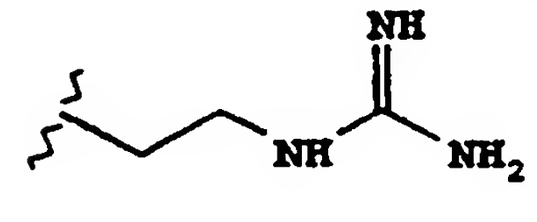

(spermidine)

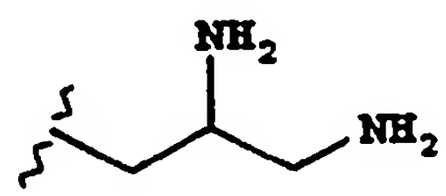
(n = 1-10)


(n = 1-10)


(n = 0-10)




(guanidine)



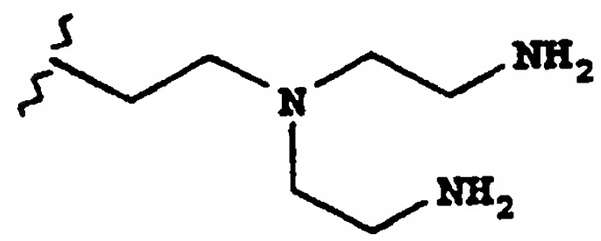
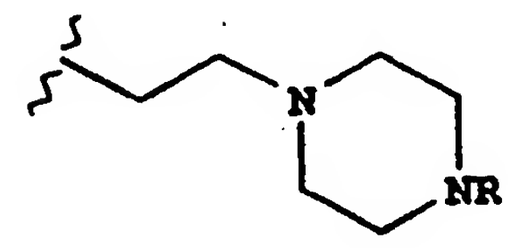
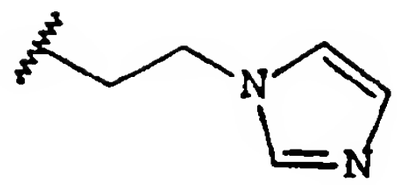
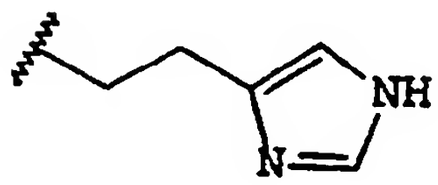
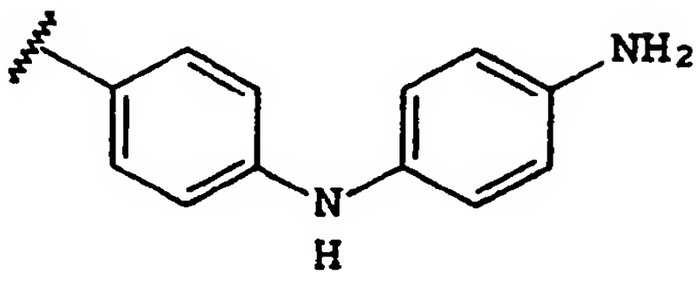


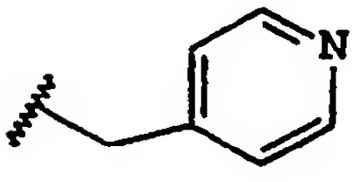
FIG. 3

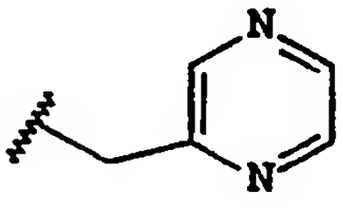

(n = 0-10; R = H or poly(aminoalkylene)

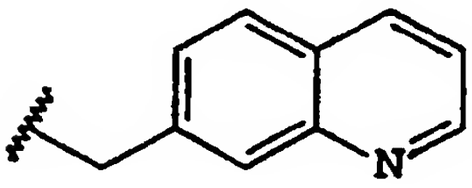












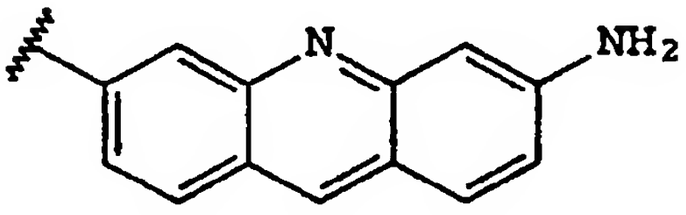


FIG. 4

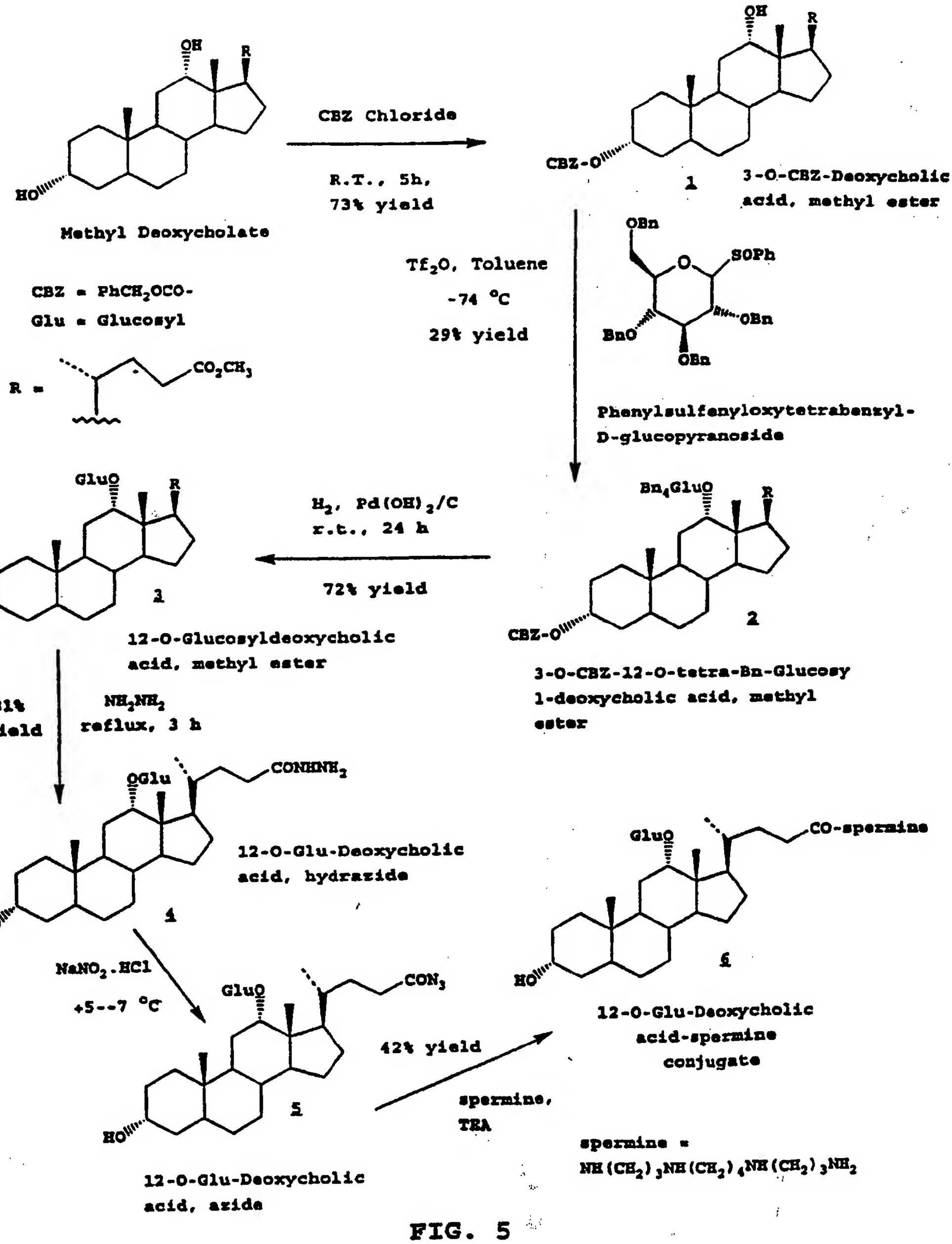
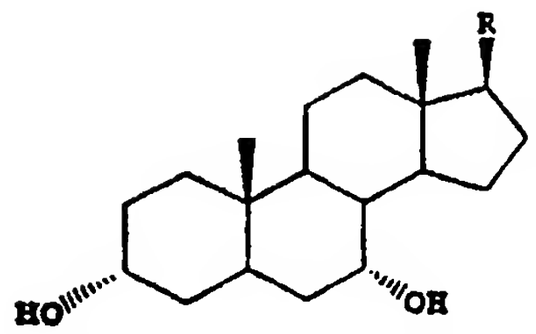

OH
R
HO
Methyl Deoxycholate
CBZ Chloride
R.T., 5h,
73% yield
CBZ-O
1
3-O-CBZ-Deoxycholic acid, methyl ester
CBZ = PhCH2OCO-
Glu = Glucosyl
R =
CO2CH3
Tf2O, Toluene
-74 °C
29% yield
OBn
SOPh
BnO
OBn
OBn
Phenylsulfenyloxytetrabenzyl-D-glucopyranoside
GluO
R
HO
3
H2, Pd(OH)2/C
r.t., 24 h
72% yield
Bn4GluO
R
CBZ-O
2
12-O-Glucosyldeoxycholic acid, methyl ester
3-O-CBZ-12-O-tetra-Bn-Glucosy 1-deoxycholic acid, methyl ester
81% yield
NH2NH2
reflux, 3 h
OGlu
CONHNH2
HO
4
12-O-Glu-Deoxycholic acid, hydrazide
NaNO2.HCl
+5--7 °C
GluO
CON3
HO
5
42% yield
spermine, TEA
GluO
CO-spermine
HO
6
12-O-Glu-Deoxycholic acid-spermine conjugate
12-O-Glu-Deoxycholic acid, azide
spermine =
NH(CH2)3NH(CH2)4NH(CH2)3NH2

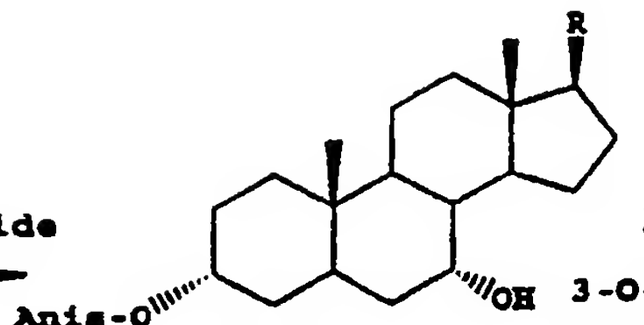
FIG. 5



Methyl Chenodeoxycholate

Anisoyl Chloride

78% yield

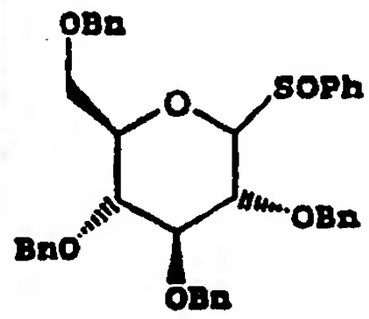


1

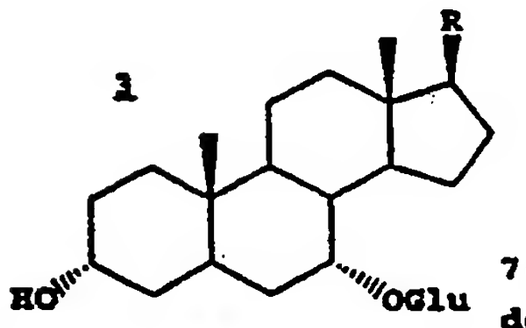
3-O-Anis-Chenodeoxy cholic acid, methyl ester

Anis = p-$CH_3OC_6H_4CO$-
Glu = Glucosyl

Tf_2O, Base
-78 °C
40% yield



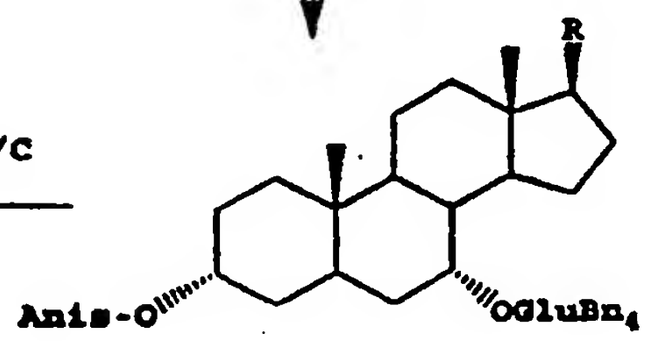
Phenylsulfenyloxytetrabenzyl-D-glucopyranoside



1

7-O-Glucosylchenodeoxycholic acid, methyl ester

H_2, $Pd(OH)_2/C$

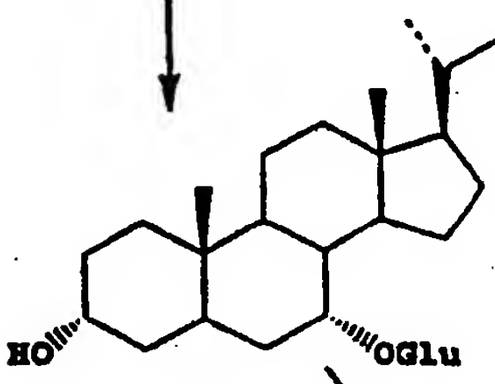


2

3-O-Anis-7-O-tetra-Bn-Glucosyl-chenodeoxycholic acid, methyl ester

75% yield

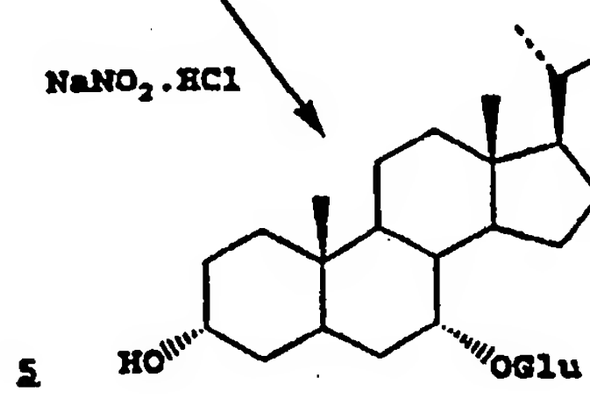
NH_2NH_2, EtOH
reflux, 3 h



7-O-Glu-Chenodeoxy cholic acid, hydrazide

4

$NaNO_2$.HCl



5

7-O-Glu-Chenodeoxy cholic acid, azide

70% yield

spermine,
TEA, r.t.
30 min

6

7-O-Glu-Chendeoxycholic acid-spermine conjugate

spermine =
$NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

FIG. 6

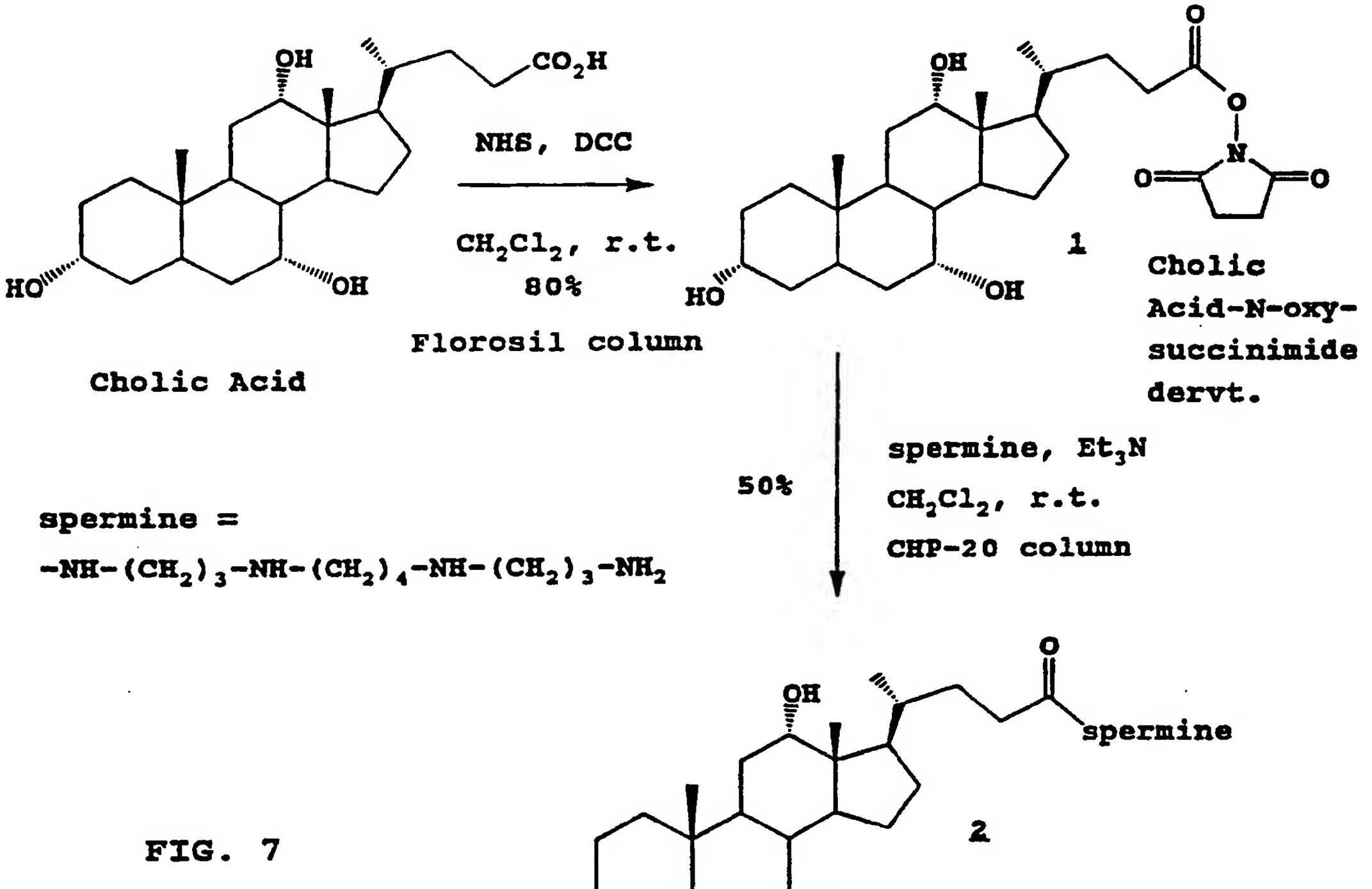


FIG. 7

Cholic acid-spermine conjugate

BNSDOCID: <WO_____9529186A1_I_>

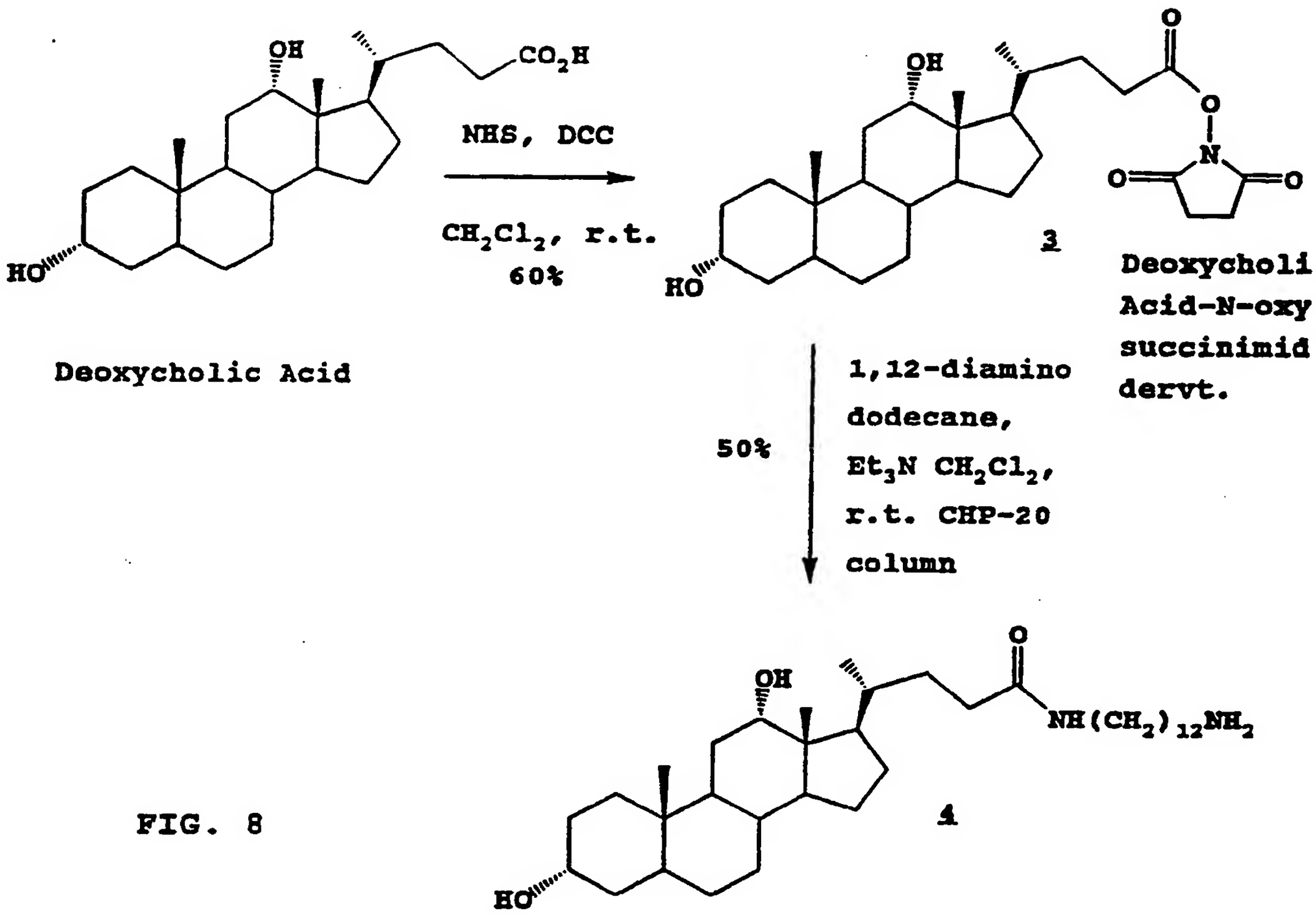
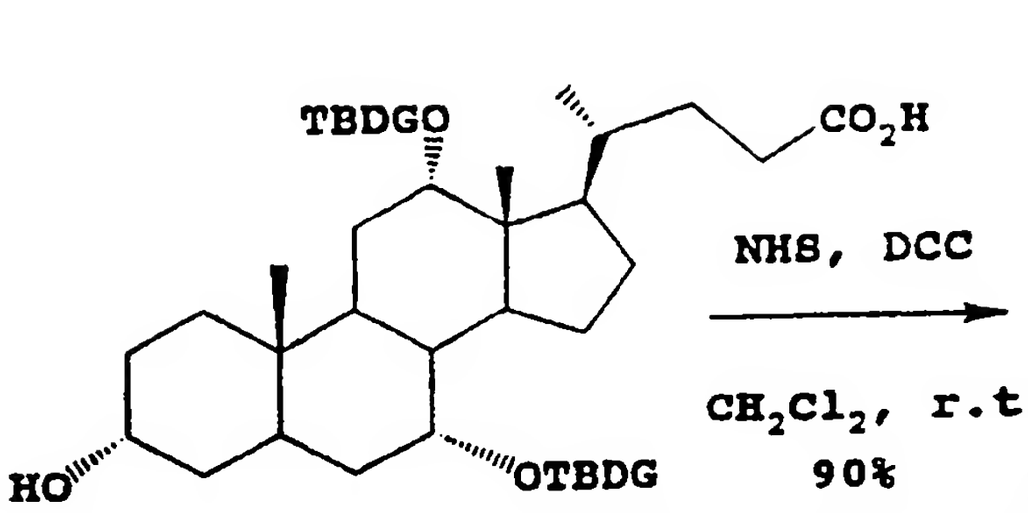


FIG. 8

BNSDOCID: <WO_____9529186A1_I_>



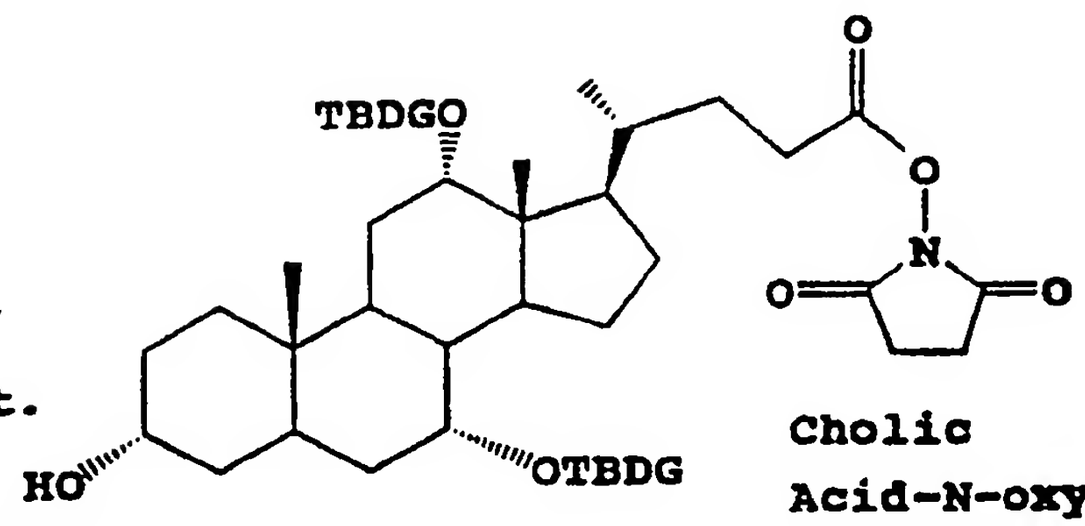
NHS, DCC

CH_2Cl_2, r.t.

90%

Florosil column

7,12-Bis-α-perbenzyl glucosylcholic Acid



5

Cholic Acid-N-oxy-succinimide dervt.

TBDG =

tetra-O-benzyl-α-D-glucopyranose

85%

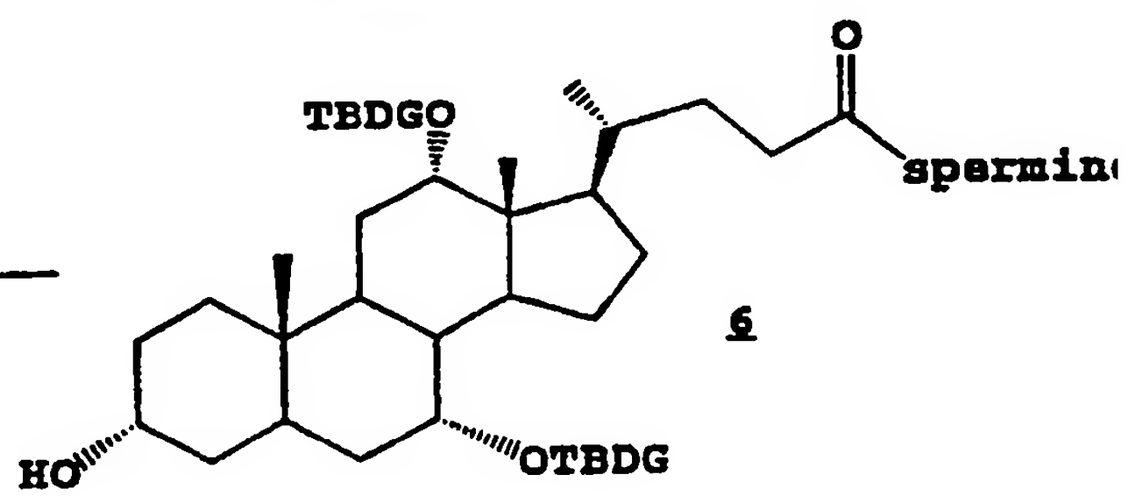
spermine, Et_3N

CH_2Cl_2, r.t.

CHP-20 column

spermine =

$-NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$



6

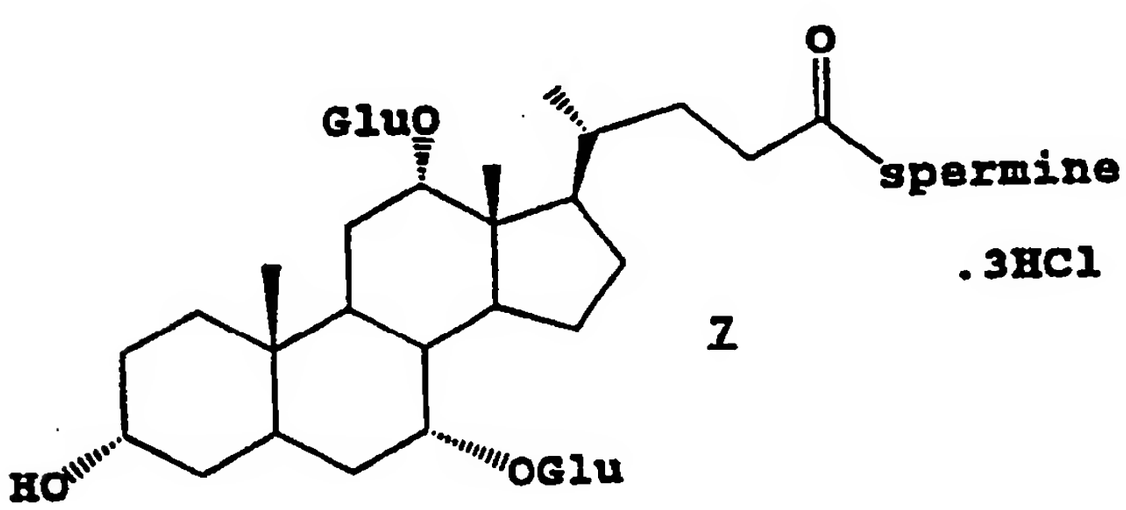
Bis(OTBDG)cholic acid-spermine conjugate

$Pd(OH)_2/C$, H_2 (50 psi)

$THF:H_2O$ 2:1, 1N HCl

CHP-20 column

70% yield

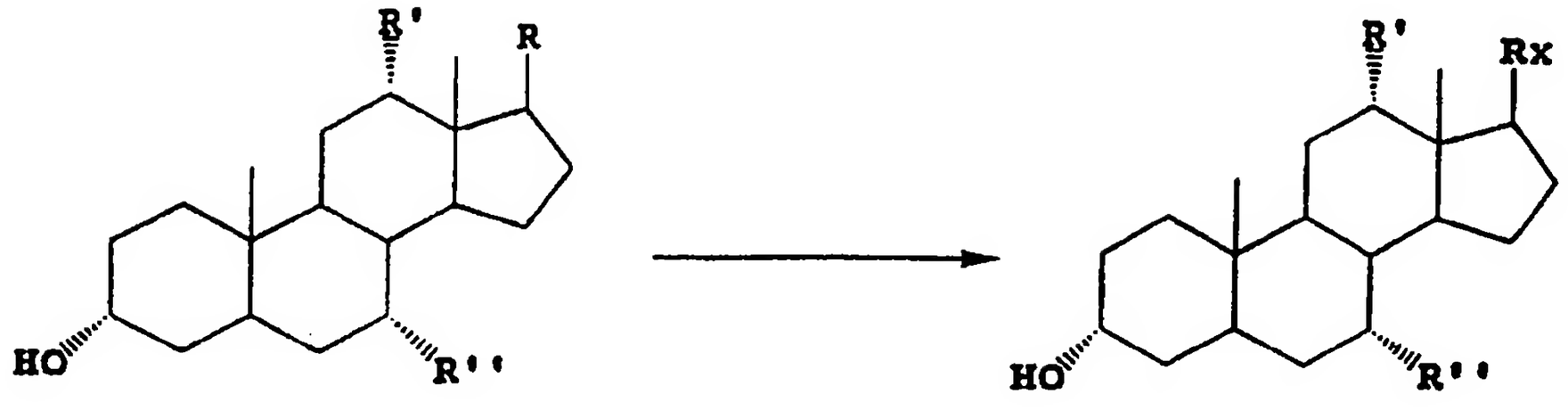


7

.3HCl

Bis(glycosylated)cholic acid-spermine conjugate

FIG. 9



R = $-CH(CH_3)CH_2CH_2CO_2N$-succinyl

Compound	R'	R''	Rx
1	-OH	-H	$-CH(CH_3)CH_2CH_2CONH(CH_2CH_2NH)_3CH_2CH_2NH_2$
2	-OH	-H	$-CH(CH_3)CH_2CH_2CONH(CH_2CH_2NH)_4CH_2CH_2NH_2$
3	-H	-OH	$-CH(CH_3)CH_2CH_2CONH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

FIG. 10

BNSDOCID: <WO 9529186A1 I >

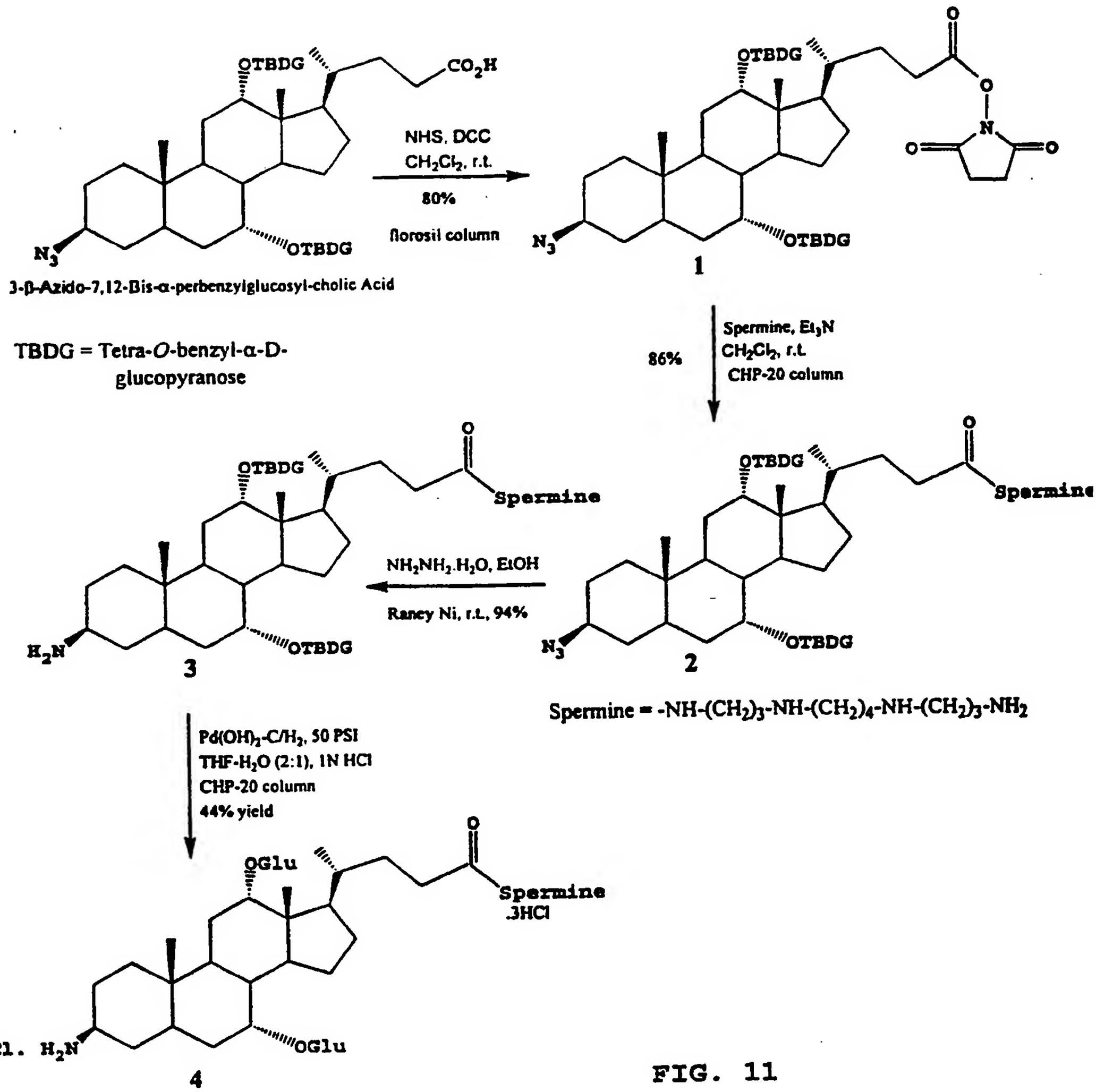
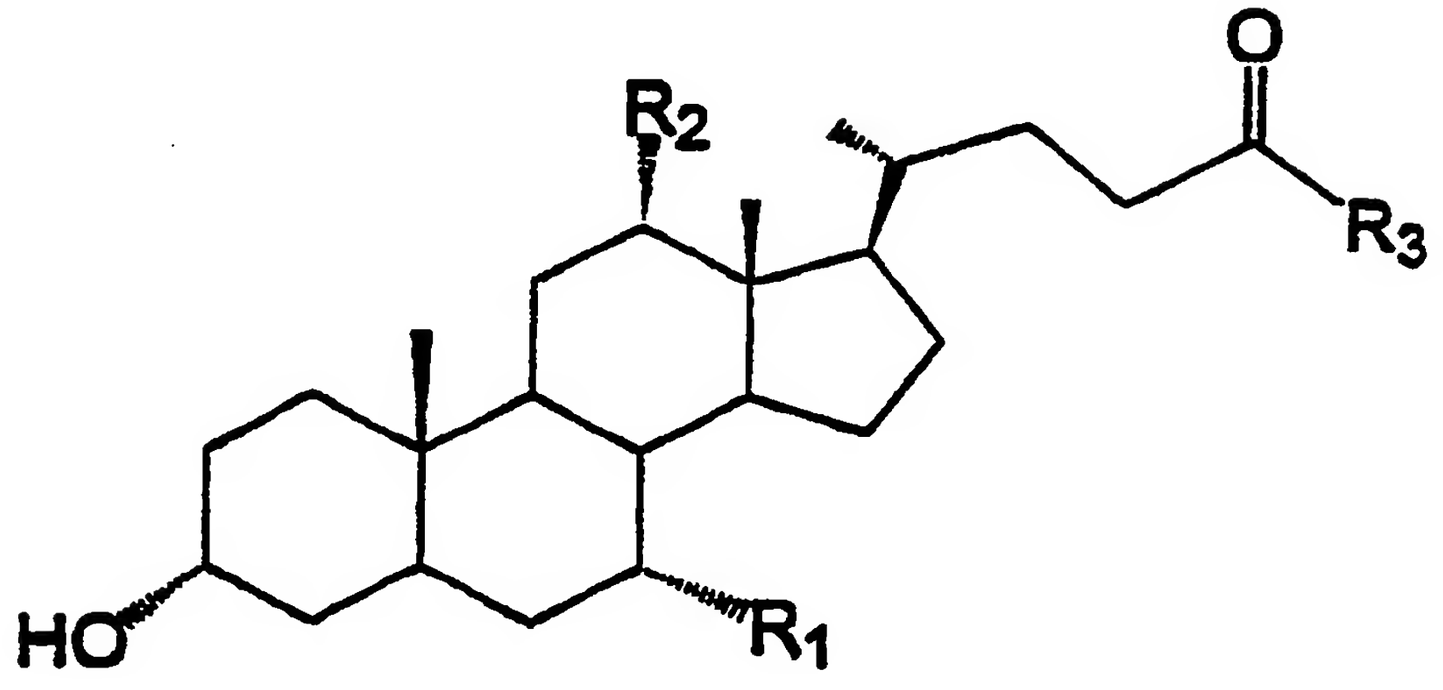


FIG. 11

BNSDOCID: <WO_____9529186A1_I_>

FIG. 12

Compounds Used in the Transportation Study of pSVβ Plasmid for Expressing β-Galactosidase Gene in COS-7 Cells



1 $R_1 = R_2 =$ O-α–D-glucopyranose; $R_3 =$ OCH_3

(F) 2 $R_1 = R_2 =$ OH; $R_3 =$ *Spermine

(O) 3 $R_1 =$ H; $R_2 =$ O-α–D-glucopyranose; $R_3 =$ *Spermine

(P) 4 $R_1 =$ O-α–D-glucopyranose; $R_2 =$ H; $R_3 =$ *Spermine

(E) 5 $R_1 = R_2 =$ O-α–D-glucopyranose; $R_3 =$ *Spermine

(N) 6 $R_1 = R_2 =$ O-α–D-glucopyranose;

$R_3 = -NH-(CH_2-CH_2-NH)_3-(CH_2)_2-NH_2$

(M) 7 $R_1 = R_2 =$ O-α–D-glucopyranose;

$R_3 = -NH-(CH_2-CH_2-NH)_4-(CH_2)_2-NH_2$

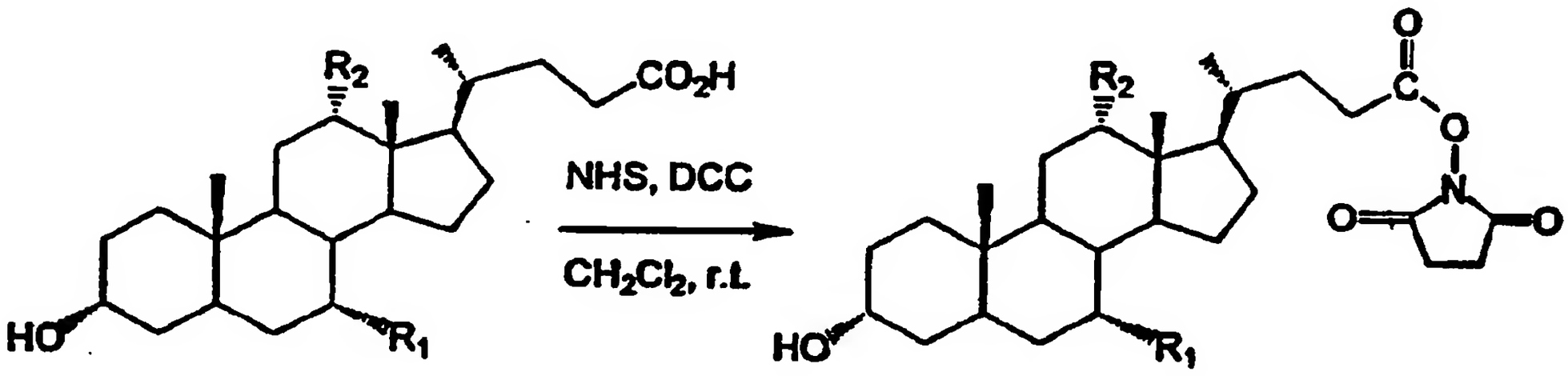
*Spermine = $-NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$

BNSDOCID: <WO_____9529186A1_I_>

FIG. 13

Synthesis of Glycosylated-Steroid Polyamine conjugates

Scheme 1



8 $R_1 = R_2 = OH$

***9** $R_1 = R_2 =$ O-α -tetra-*O*-benzyl-D-glucose

*For synthesis see Reference 10 and 11

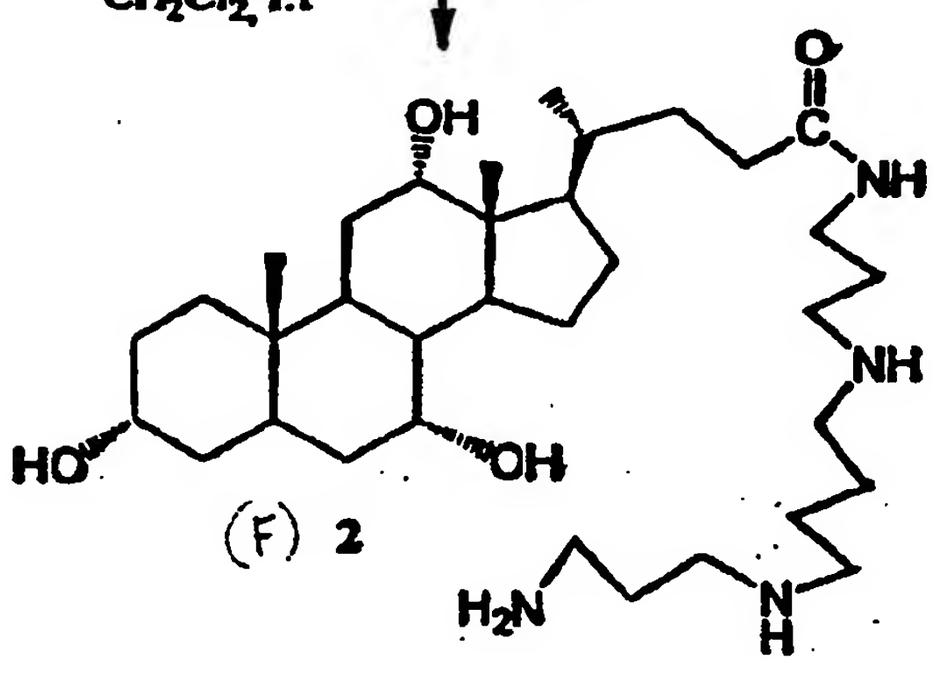
10 $R_1 = R_2 = OH$

11 $R_1 = R_2 =$ O-α -tetra-*O*-benzyl-D-glucose

10

Spermine, Et_3N
CH_2Cl_2, r.t

50%

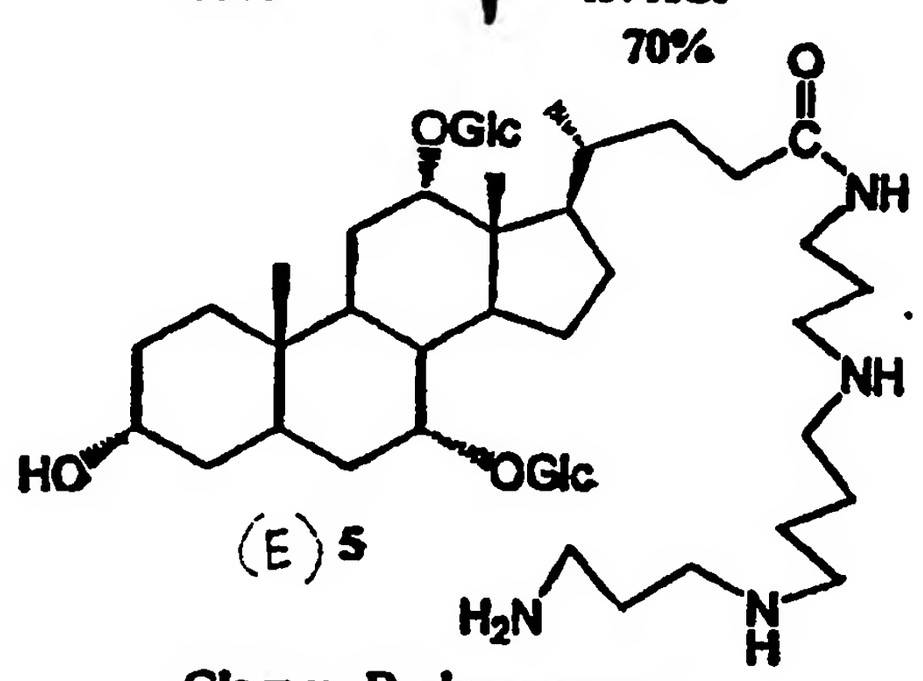


(F) **2**

11

1) Spermine, Et_3N
CH_2Cl_2, r.t
85%

2) $PdOH_2$-C / H_2,
THF:H_2O (2:1),
1N HCl
70%



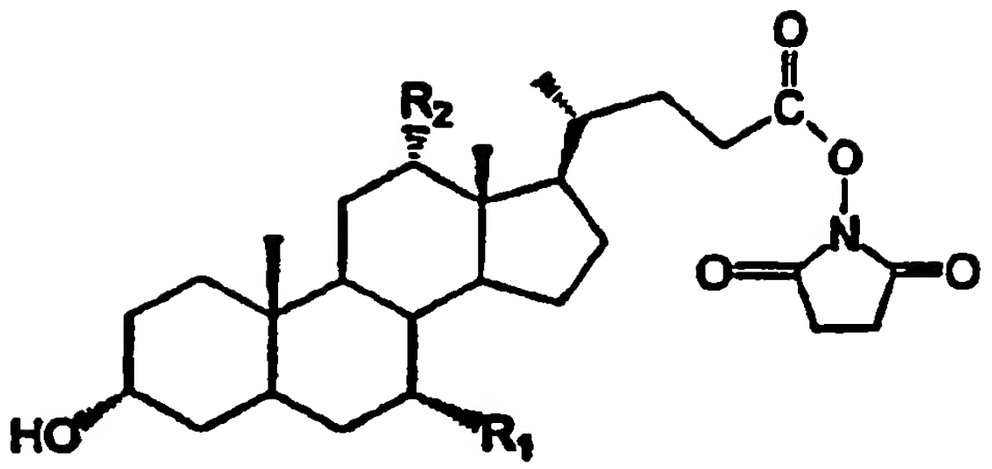
(E) **5**

Glc = α -D-glucopyranose

BNSDOCID: <WO_____9529186A1_I_>

FIG. 14

Scheme 2



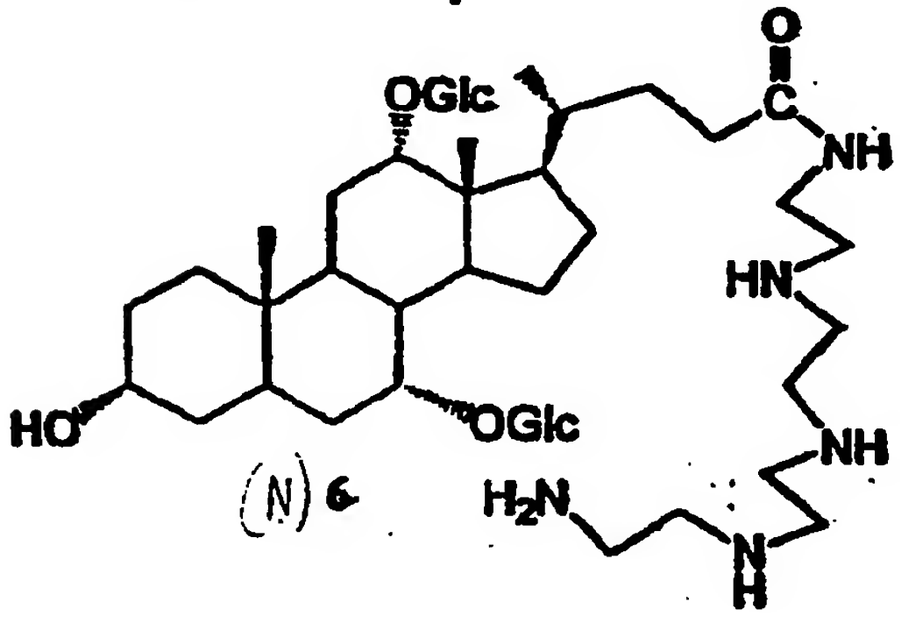
11 $R_1 = R_2$ = O-α -tetra-*O*-benzyl-D-glucose

1) Tetraethylene-pentamine , Et_3N, CH_2Cl_2 r.t 64%

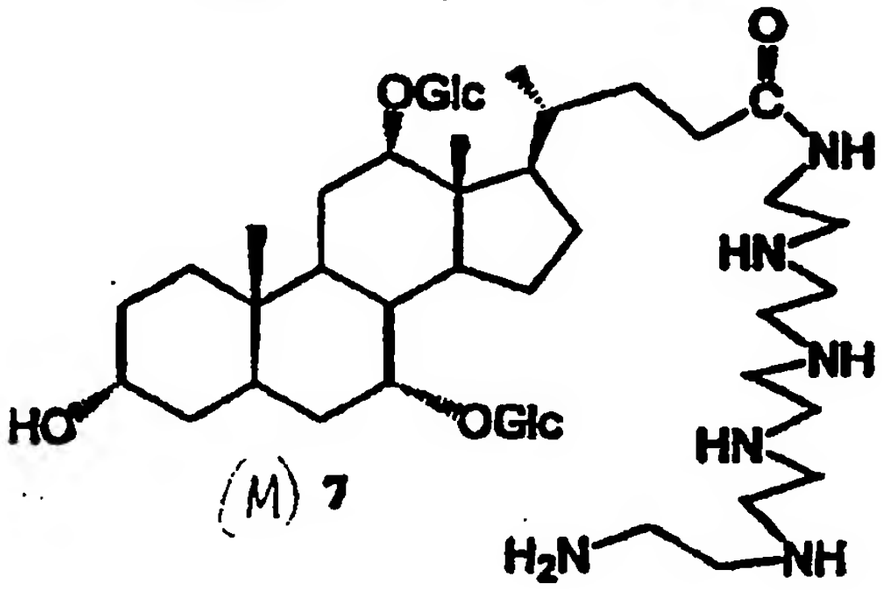
2) $PdOH_2$-C / H_2, THF:H_2O (2:1), 1N HCl 51%

1) Pentaethylene-Hexamine , Et_3N, CH_2Cl_2 r.t 57%

2) $PdOH_2$-C / H_2, THF:H_2O (2:1), 1N HCl 50%



(N) 6



(M) 7

Glc = α -D-glucopyranose

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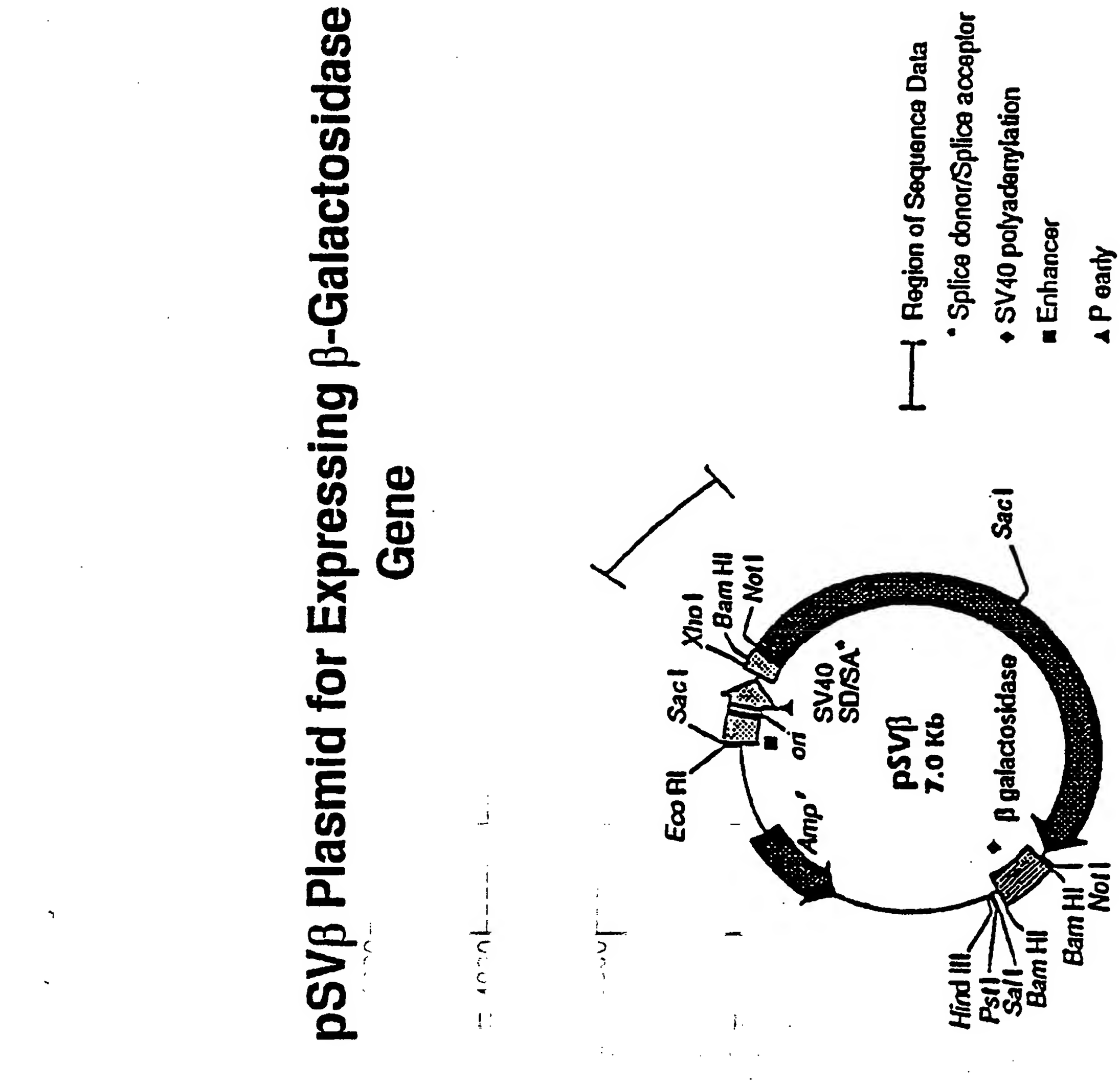
FIG. 15


pSVβ Plasmid for Expressing β-Galactosidase Gene
Region of Sequence Data
Splice donor/Splice acceptor
SV40 polyadenylation
Enhancer
P early
pSVβ
7.0 Kb
SV40 SD/SA
β galactosidase
Sac I
Xho I
Bam HI
Not I
ori
Eco RI
Amp'
Hind III
Pst I
Sal I
Bam HI
Bam HI
Not I
Sac I

BNSDOCID: <WO_____9529186A1 | >

Results of Transfections with Transcell Compounds

Compound No.	Polyamine Side Chain	Ratio Cmpd / DNA Charge	Ratio Cmpd / DNA Mole	% Transfection Relative to Lipofectin
2 (F)	$-NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$	25	17	27
3 (O)	$-NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$	24	16	20
4 (P)	$-NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$	24	16	207
5 (E)	$-NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$	96	64	380
6 (H)	$-NH-(CH_2-CH_2-NH)_3-CH_2-CH_2-NH_2$	96	64	127
7 (M)	$-NH-(CH_2-CH_2-NH)_5-CH_2-CH_2-NH_2$	58	24	1300

FIG. 17

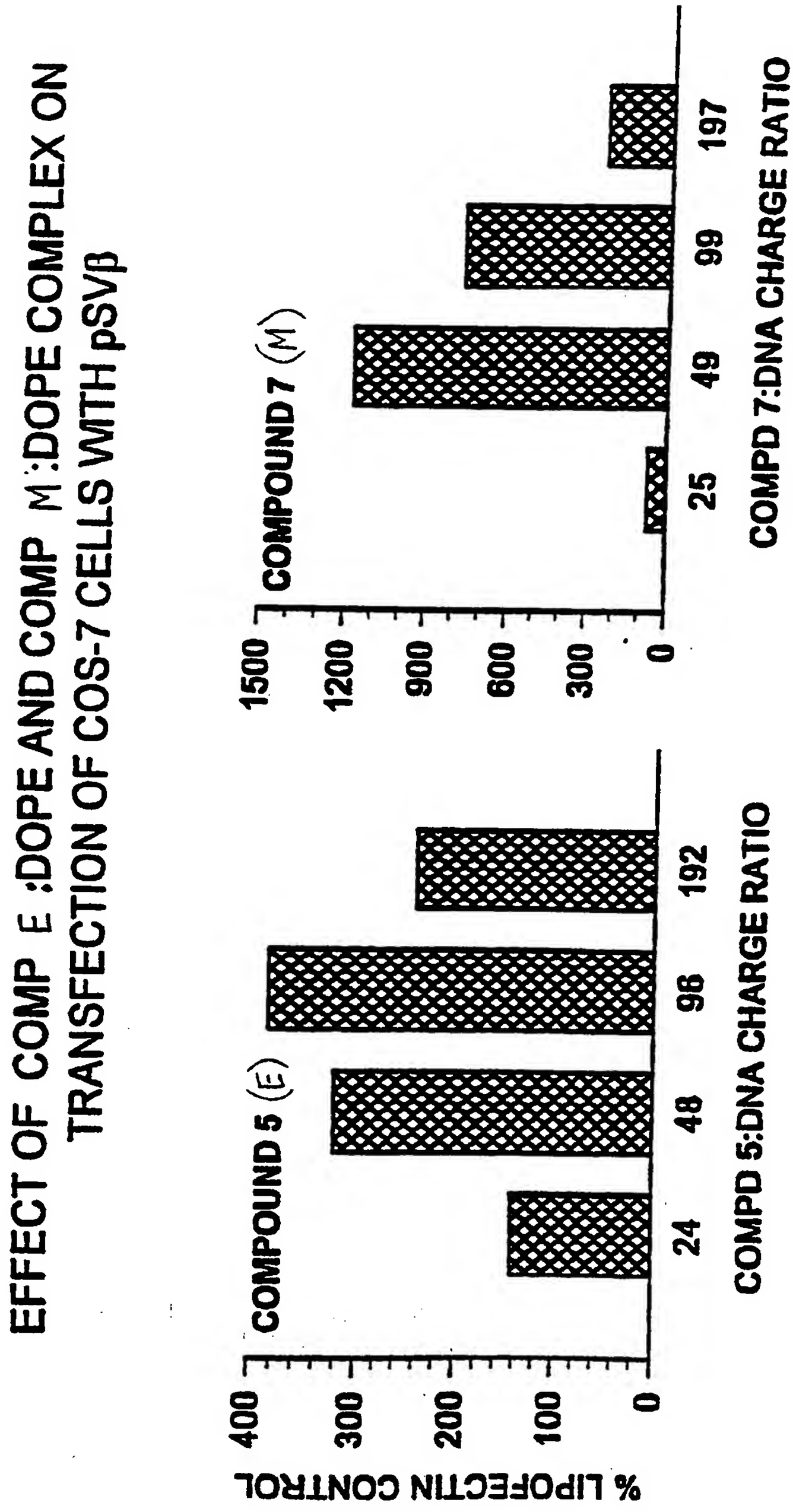


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Fig. 18

Comparison of ONPG and X-GAL Assays for Compound M:DOPE Complex

% LIPOFECTIN CONTROL			CHARGE RATIO (COMPOUND:DNA)
ONPG	X-GAL		
11	7.8		3
23	7.8		6
14	10.5		12
88	63.1		26
1216	1241		49
811	350		99
318	68.4		197
84	62		394

The level of β-Galactosidase expression increases concurently with transfection frequency.

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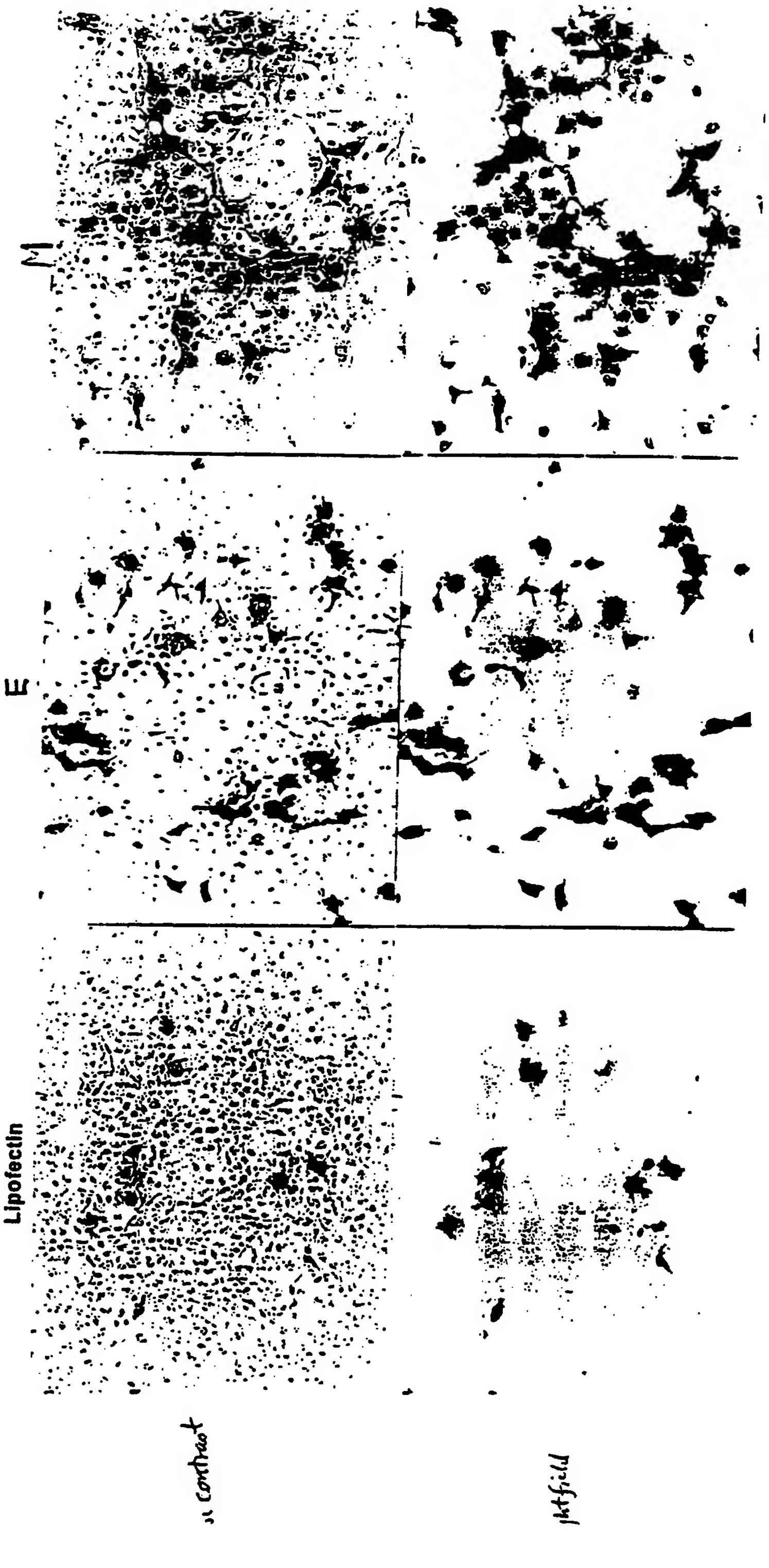

Transfection of COS-7 Cells with pSVβ Using Glycosterold Polyamine:DOPE Complex
Lipofectin
E
1 contact
Intfield

Fig. 19

Fig. 20

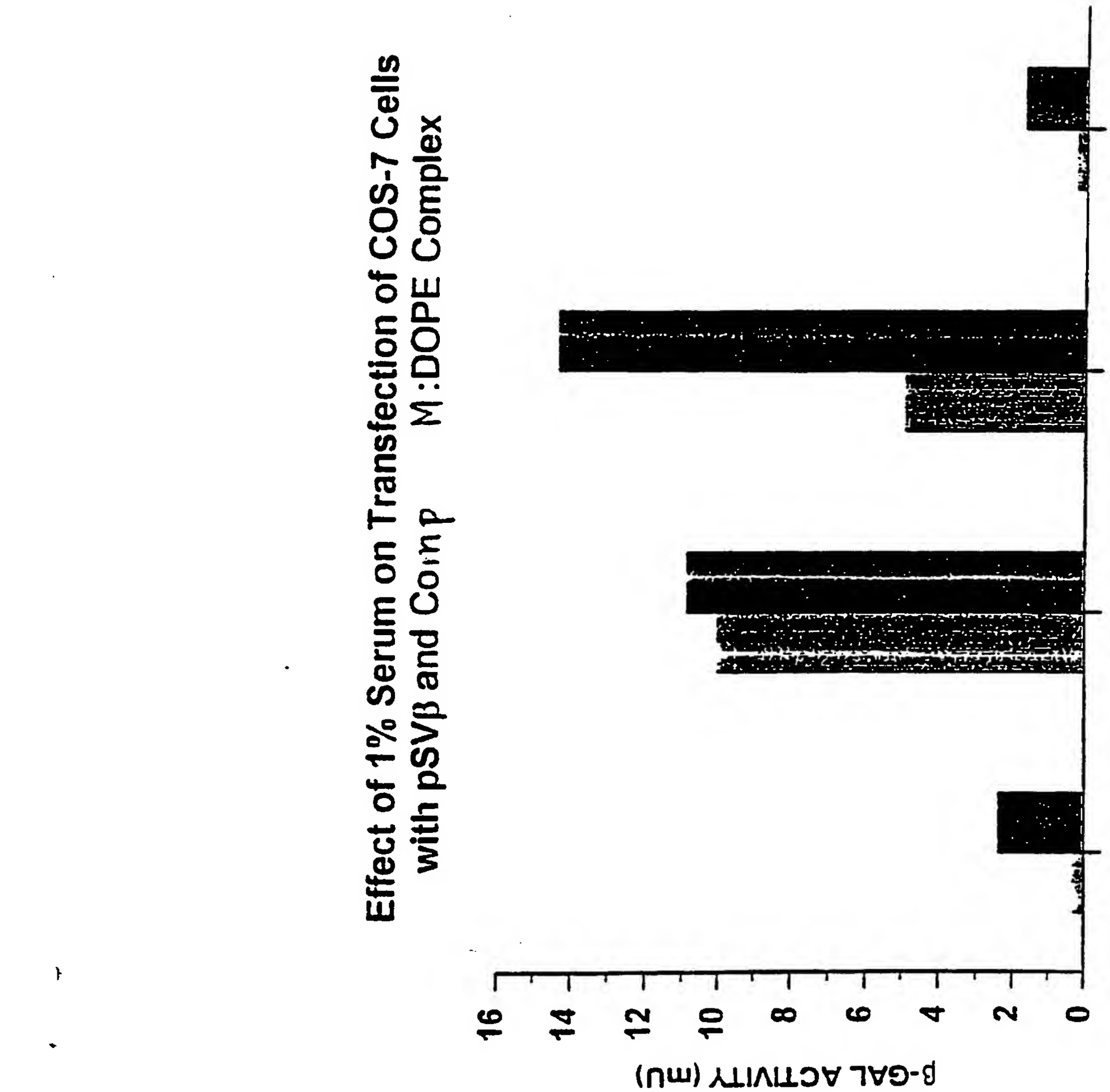
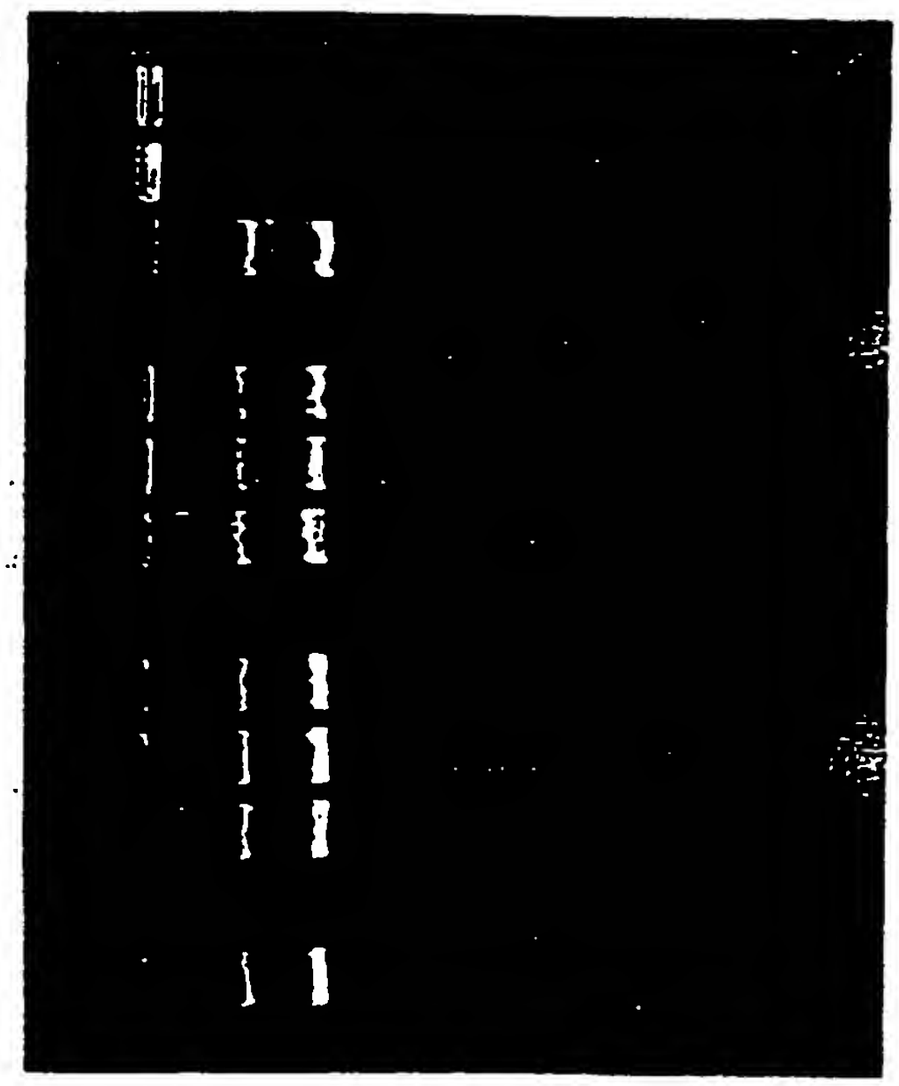

Effect of 1% Serum on Transfection of COS-7 Cells with pSVβ and Comp M:DOPE Complex
β-GAL ACTIVITY (mU)
0
2
4
6
8
10
12
14
16
POLYAMINE M:DNA Charge Ratio
25
49
99
WITHOUT SERUM
WITH 1% SERUM

Fig. 21

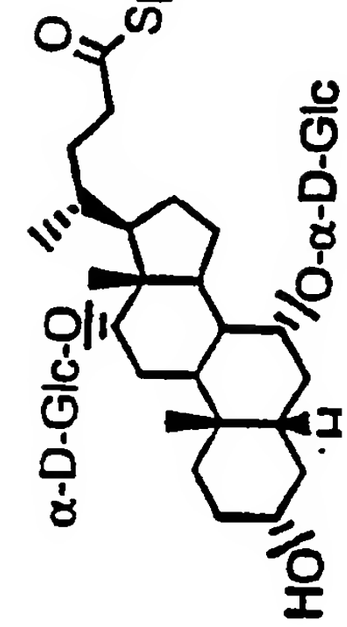
Gel Shift Experiment / DNA Binding

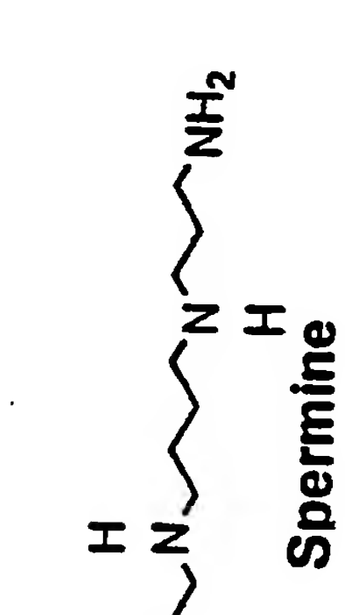
Electrophoretic Migration of pBR322 DNA

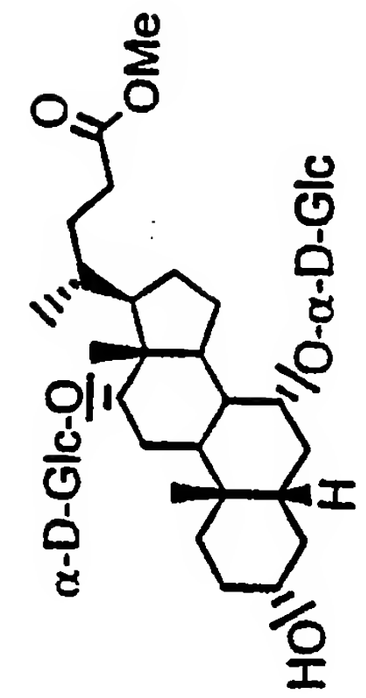
Cmpd 1 Spermine Cmpd E



Conditions:
a) 0.25µg DNA
b) All cmpds formulated with 1:1 w:w DOPE







BNSDOCID: <WO_____9529186A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04806

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :514/26, 44; 536/5, 22.1, 23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/26, 44; 536/5, 22.1, 23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,994,439 (LONGENECKER ET AL.) 19 February 1991 (see entire document).	49-59
A	US, A, 5,122,520 (AZRIA ET AL.) 16 June 1992 (see entire document).	49-59
A	US, A, 5,273,965 (KENSIL ET AL.) 28 December 1993 (see entire document).	1-26, 48, 60, 61, 68 and 69
P, X	US, A, 5,338,837 (KAHNE) 16 August 1994 (see entire document).	27-38, 62 and 63
Y	WO, A, 91/14696 (LATHAM ET AL.) 03 October 1991 (see entire document).	1-26, 39-61 and 64-69

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 JULY 1995	01 AUG 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer HOWARD C. LEE Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

BNSDOCID: <WO_____9529186A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04806

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, USA, Volume 86, issued September 1989, Letsinger et al., "Cholesteryl-conjugated oligonucleotides: Synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture", pages 6553-6556, see entire document.	1-26, 39-61 and 64-69
Y	Proceedings of the National Academy of Sciences, USA, Volume 82, issued November 1985, Gordon et al. "Nasal absorption of insulin: Enhancement by hydrophobic bile salts", pages 7419-7423, see entire document.	49-59
Y	The Journal of Biological Chemistry, Volume 267, Number 26, issued 15 September 1992, Kramer et al., "Liver-specific Drug Targeting by Coupling to Bile Acids", pages 18598-18604, see entire document.	49-59

BNSDOCID: <WO_____9529186A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04806

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

RNSDOCID: <WO 9529186A1 >

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04806

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07J 3/00, 5/00, 7/00; A61K 31/70, 31/705; C07H 19/00, 21/00, 21/02, 21/04

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, CA, EMBASE, MEDLINE, WPIDS
search terms: drug transport/targeting/delivery, nucleic acid, nucleotide, oligonucleotide, DNA, RNA, gene, triterpene, saponin, cholesterol, steroid, bile acid

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-26, 39-48, 60, 61 and 64-69 are, drawn to steroid/steroid-glycoside, nucleic acid and fusogenic lipid compositions and the use of such compositions for the introduction of nucleic acids into a host cell and transforming the host cell.

Group II, claims 27-38, 49-59, 62 and 63 are, drawn to steroid/steroid glycoside derivative compounds.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

A. DNA/RNA which encodes a gene which corresponds to the claims of Group I, specifically claims 3, 4, 25, 26 and 46.
B. DNA/RNA which encodes an anti-sense oligonucleotide which corresponds to the claims of Group I, specifically claim 47.

The claims are deemed to correspond to the species listed above in the following manner:
The claims of Group I read broadly upon a multitude of DNA/RNA sequences and functions.The following claims are generic: 1, 5-20, 39-45, 48-60, 61 and 64-69.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The claims of Group I are directed toward a multicomponent composition and the use thereof while the claims of Group II are directed toward one of those components. However, the special technical feature of the claims of Group I is the introduction of nucleic acids and the ability of those nucleic acids to transform a cell. However, the special technical feature of the steroid/steroid-glycoside compound alone (i.e without nucleic acids) is not the same as that disclosed for the claims of Group I.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Each of the species of DNA/RNA have specific functions which do not share a special technical feature. Anti-sense RNA are frequently used to act as a repressor of normal function or expression of targeted DNA while anti-sense DNA pair with and inactivate complementary mRNA sequences for the purposes of control and regulation. The encoding of genes or modification of the encoding of genes entails the modification of the species genome through gene targeting, homologous recombination and other genomic modification techniques such as replacement, mutation or insertion and as such said activity would not be seen as related to anti-sense function. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

BNSDOCID: <WO_____9529186A1_I_>